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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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TITLE OF THE INVENTION (500 characters max)

GLYCOCONJUGATION USING SACCHARYL FRAGMENTS

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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☒ Application Data Sheet. See 37 CFR 1.76

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Respectfully submitted,

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PROVISIONAL

PATENT APPLICATION

GLYCOCONJUGATION USING SACCHARYL FRAGMENTS

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AS FILED IN THE USPTO ON JANUARY 6, 2005

GLYCOCONJUGATION USING SACCHARYL FRAGMENTS

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present invention relates to conugates formed between a biologically relevant substrate (e.g., a glycosylated or non-glycosylated peptide or lipid) and a saccharyl fragment that includes a modifying group ("modified fragment"). The substrate and modified fragment are linked through an enzymatically formed bond between the modified fragment and an acceptor moiety on the substrate.

Background

[0002] The administration of glycosylated and non-glycosylated therapeutic agents for engendering a particular physiological response is well known in the medicinal arts. For example, both purified and recombinant hGH are used for treating conditions and diseases due to hGH deficiency, *e.g.*, dwarfism in children, interferon has known antiviral activity and granulocyte colony stimulating factor stimulates the production of white blood cells.

[0003] A principal factor that has limited the use of therapeutic peptides is the difficulty inherent in engineering an expression system to express a peptide having the glycosylation pattern of the wild-type peptide. Improperly or incompletely glycosylated peptides can be immunogenic; in a patient, an immunogenic response to an administered peptide can neutralize the peptide and/or lead to the development of an allergic response in the patient. Other deficiencies of recombinantly produced glycopeptides include suboptimal potency and rapid clearance rates. The problems inherent in peptide therapeutics are recognized in the art, and various methods of eliminating the problems have been investigated.

[0004] Post-expression *in vitro* modification of peptides is an attractive strategy to remedy the deficiencies of methods that rely on controlling glycosylation by engineering expression systems; including both modification of glycan structures or introduction of glycans at novel sites. A comprehensive toolbox of recombinant eukaryotic glycosyltransferases is becoming available, making *in vitro* enzymatic synthesis of mammalian glycoconjugates with custom designed glycosylation patterns and glycosyl structures possible. See, for example, U.S. Patent No. 5,876,980; 6,030,815; 5,728,554; 5,922,577; and WO/9831826; US2003180835; and WO 03/031464.

[0005] Enzyme-based syntheses have the advantages of regioselectivity and stereoselectivity. Moreover, enzymatic syntheses are performed using unprotected substrates. Two principal classes of enzymes are used in the synthesis of carbohydrates, glycosyltransferases (*e.g.*, sialyltransferases, oligosaccharyltransferases, N-acetylglucosaminyltransferases), and glycosidases. The glycosidases are further classified as exoglycosidases (*e.g.*, β-mannosidase, β-glucosidase), and endoglycosidases (*e.g.*, Endo-A, Endo-M). Each of these classes of enzymes has been successfully used synthetically to prepare carbohydrates. For a general review, *see,* Crout *et al., Curr. Opin. Chem. Biol.* **2**: 98-111 (1998).

[0006] Glycosyltransferases modify the oligosaccharide structures on glycopeptides. Glycosyltransferases are effective for producing specific products with good stereochemical and regiochemical control. Glycosyltransferases have been used to prepare oligosaccharides and to modify terminal N- and O-linked carbohydrate structures, particularly on glycopeptides produced in mammalian cells. For example, the terminal oligosaccharides of glycopeptides have been completely sialylated and/or fucosylated to provide more consistent sugar structures, which improves glycopeptide pharmacodynamics and a variety of other biological properties. For example, β-1,4-galactosyltransferase was used to synthesize lactosamine, an illustration of the utility of glycosyltransferases in the synthesis of carbohydrates (*see, e.g.*, Wong *et al., J. Org. Chem.* **47**: 5416-5418 (1982)). Moreover, numerous synthetic procedures have made use of α-sialyltransferases to transfer sialic acid from cytidine-5'-monophospho-N-acetylneuraminic acid to the 3-OH or 6-OH of galactose (*see, e.g.*, Kevin *et al., Chem. Eur. J.* **2**: 1359-1362 (1996)). Fucosyltransferases are used in synthetic pathways to transfer a fucose unit from guanosine-5'-diphosphofucose to a specific hydroxyl of a saccharide acceptor. For example, Ichikawa prepared sialyl Lewis-X by a method that involves the fucosylation of sialylated lactosamine with a cloned fucosyltransferase (Ichikawa *et al., J. Am. Chem. Soc.* **114**: 9283-9298 (1992)). For a discussion of recent advances in glycoconjugate synthesis for therapeutic use *see,* Koeller *et al., Nature Biotechnology* **18**: 835-841 (2000). *See* also, U.S. Patent No. 5,876,980; 6,030,815; 5,728,554; 5,922,577; and WO/9831826.

[0007] Glycosidases can also be used to prepare saccharides. Glycosidases normally catalyze the hydrolysis of a glycosidic bond. Under appropriate conditions, however, they can be used to form this linkage. Most glycosidases used for carbohydrate synthesis are exoglycosidases; the glycosyl transfer occurs at the non-reducing terminus of the substrate.

The glycosidase takes up a glycosyl donor in a glycosyl-enzyme intermediate that is either intercepted by water to give the hydrolysis product, or by an acceptor, to give a new glycoside or oligosaccharide. An exemplary pathway using an exoglycosidase is the synthesis of the core trisaccharide of all N-linked glycopeptides, including the difficult β-mannoside linkage, which was formed by the action of β-mannosidase (Singh *et al., Chem. Commun.* 993-994 (1996)).

[0008] In another exemplary application of the use of a glycosidase to form a glycosidic linkage, a mutant glycosidase was prepared in which the normal nucleophilic amino acid within the active site is changed to a non-nucleophilic amino acid. The mutant enzymes do not hydrolyze glycosidic linkages, but can still form them. The mutant glycosidases are used to prepare oligosaccharides using an α-glycosyl fluoride donor and a glycoside acceptor molecule (Withers *et al.*, U.S. Patent No. 5,716,812). Although the mutant glycosidases are useful for forming free oligosaccharides, it has yet to be demonstrated that such enzymes are capable of appending glycosyl donors onto glycosylated or non-glycosylated peptides, nor have these enzymes been used with unactivated glycosyl donors.

[0009] Although their use is less common than that of the exoglycosidases, endoglycosidases are also utilized to prepare carbohydrates. Methods based on the use of endoglycosidases have the advantage that an oligosaccharide, rather than a monosaccharide, is transferred. Oligosaccharide fragments have been added to substrates using *endo*-β-N-acetylglucosamines such as *endo*-F, *endo*-M (Wang *et al., Tetrahedron Lett.* **37**: 1975-1978); and Haneda *et al., Carbohydr. Res.* **292**: 61-70 (1996)).

[0010] In addition to their use in preparing carbohydrates, the enzymes discussed above are applied to the synthesis of glycopeptides. The synthesis of a homogenous glycoform of ribonuclease B has been published (Witte K. *et al., J. Am. Chem. Soc.* **119**: 2114-2118 (1997)). The high mannose core of ribonuclease B was cleaved by treating the glycopeptide with endoglycosidase H. The cleavage occurred specifically between the two core GlcNAc residues. The tetrasaccharide sialyl Lewis X was then enzymatically rebuilt on the remaining GlcNAc anchor site on the now homogenous protein by the sequential use of β-1,4-galactosyltransferase, α-2,3-sialyltransferase and α-1,3-fucosyltransferase V. Each enzymatically catalyzed step proceeded in excellent yield.

[0011] Methods combining both chemical and enzymatic synthetic elements are also known. For example, Yamamoto and coworkers (*Carbohydr. Res.* **305**: 415-422 (1998))

reported the chemoenzymatic synthesis of the glycopeptide, glycosylated Peptide T, using an endoglyosidase. The N-acetylglucosaminyl peptide was synthesized by purely chemical means. The peptide was subsequently enzymatically elaborated with the oligosaccharide of human transferrin glycopeptide. The saccharide portion was added to the peptide by treating it with an endo-β-N-acetylglucosaminidase. The resulting glycosylated peptide was highly stable and resistant to proteolysis when compared to the peptide T and N-acetylglucosaminyl peptide T.

[0012] The use of glycosyltransferases to modify peptide structure with reporter groups has been explored. For example, Brossmer *et al.* (U.S. Patent No. 5,405,753) discloses the formation of a fluorescent-labeled cytidine monophosphate ("CMP") derivative of sialic acid and the use of the fluorescent glycoside in an assay for sialyl transferase activity and for the fluorescent labeling of cell surfaces, glycoproteins and gangliosides. Gross *et al.* (*Analyt. Biochem.* **186**: 127 (1990)) describe a similar assay. Bean *et al.* (U.S. Patent No. 5,432,059) discloses an assay for glycosylation deficiency disorders utilizing reglycosylation of a deficiently glycosylated protein. The deficient protein is reglycosylated with a fluorescent-labeled CMP glycoside. Each of the fluorescent sialic acid derivatives is substituted with the fluorescent moiety at either the 9-position or at the amine that is normally acetylated in sialic acid. The methods using the fluorescent sialic acid derivatives are assays for the presence of glycosyltransferases or for non-glycosylated or improperly glycosylated glycoproteins. The assays are conducted on small amounts of enzyme or glycoprotein in a sample of biological origin. The enzymatic derivatization of a glycosylated or non-glycosylated peptide on a preparative or industrial scale using a modified sialic acid has not been disclosed or suggested.

[0013] Considerable effort has also been directed towards the modification of cell surfaces by altering glycosyl residues presented by those surfaces. For example, Fukuda and coworkers have developed a method for attaching glycosides of defined structure onto cell surfaces. The method exploits the relaxed substrate specificity of a fucosyltransferase that can transfer fucose and fucose analogs bearing diverse glycosyl substrates (Tsuboi *et al., J. Biol. Chem.* **271**: 27213 (1996)).

[0014] The methods of modifying cell surfaces have not been applied in the absence of a cell to modify a glycosylated or non-glycosylated peptide. Moreover, the methods of cell surface modification are not utilized for the enzymatic incorporation preformed modified

glycosyl donor moiety into a peptide. Moreover, none of the cell surface modification methods are practical for producing glycosyl-modified peptides on an industrial scale.

[0015] Enzymatic methods have also been used to activate glycosyl residues on a glycopeptide towards subsequent chemical elaboration. The glycosyl residues are typically activated using galactose oxidase, which converts a terminal galactose residue to the corresponding aldehyde. The aldehyde is subsequently coupled to an amine-containing modifying group. For example, Casares *et al.* (*Nature Biotech.* **19**: 142 (2001)) have attached doxorubicin to the oxidized galactose residues of a recombinant MHCII-peptide chimera.

[0016] In addition to manipulating the structure of glycosyl groups on polypeptides, interest has developed in preparing glycopeptides that are modified with one or more non-saccharide modifying group, such as a water-soluble polymer. Poly(ethyleneglycol) ("PEG") is an exemplary polymer that has been conjugated to polypeptides. The use of PEG to derivatize peptide therapeutics has been demonstrated to reduce the immunogenicity of the peptides. For example, U.S. Pat. No. 4,179,337 (Davis *et al.*) discloses non-immunogenic polypeptides, such as enzymes and peptide hormones coupled to polyethylene glycol (PEG) or polypropylene glycol. Between 10 and 100 moles of polymer are used per mole polypeptide. Although the in vivo clearance time of the conjugate is prolonged relative to that of the polypeptide, only about 15% of the physiological activity is maintained. Thus, the prolonged circulation half-life is counterbalanced by the dramatic reduction in peptide potency.

[0017] The loss of peptide activity is directly attributable to the non-selective nature of the chemistries utilized to conjugate the water-soluble polymer. The principal mode of attachment of PEG, and its derivatives, to peptides is a non-specific bonding through a peptide amino acid residue. For example, U.S. Patent No. 4,088,538 discloses an enzymatically active polymer-enzyme conjugate of an enzyme covalently bound to PEG. Similarly, U.S. Patent No. 4,496,689 discloses a covalently attached complex of α-1 proteinase inhibitor with a polymer such as PEG or methoxypoly(ethyleneglycol) ("(m-) PEG"). Abuchowski *et al.* (*J. Biol. Chem.* **252**: 3578 (1977)) discloses the covalent attachment of (m-) PEG to an amine group of bovine serum albumin. U.S. Patent No. 4,414,147 discloses a method of rendering interferon less hydrophobic by conjugating it to an anhydride of a dicarboxylic acid, such as poly(ethylene succinic anhydride). PCT WO 87/00056 discloses conjugation of PEG and poly(oxyethylated) polyols to such proteins as

interferon-β, interleukin-2 and immunotoxins. EP 154,316 discloses and claims chemically modified lymphokines, such as IL-2 containing PEG bonded directly to at least one primary amino group of the lymphokine. U.S. Patent No. 4,055,635 discloses pharmaceutical compositions of a water-soluble complex of a proteolytic enzyme linked covalently to a polymeric substance such as a polysaccharide.

[0018] Another mode of attaching PEG to peptides is through the non-specific oxidation of glycosyl residues on a glycopeptide. The oxidized sugar is utilized as a locus for attaching a PEG moiety to the peptide. For example M'Timkulu (WO 94/05332) discloses the use of an amino-PEG to add PEG to a glycoprotein. The glycosyl moieties are randomly oxidized to the corresponding aldehydes, which are subsequently coupled to the amino-PEG.

[0019] In each of the methods described above, poly(ethyleneglycol) is added in a random, non-specific manner to reactive residues on a peptide backbone. For the production of therapeutic peptides, it is clearly desirable to utilize a derivitization strategy that results in the formation of a specifically labeled, readily characterizable, essentially homogeneous product. A promising route to preparing specifically labeled peptides is through the use of enzymes, such as glycosyltransferases to append a modified sugar moiety onto a peptide.

[0020] Glycosyl residues have also been modified to bear ketone groups. For example, Mahal and co-workers (*Science* **276**: 1125 (1997)) have prepared N-levulinoyl mannosamine ("ManLev"), which has a ketone functionality at the position normally occupied by the acetyl group in the natural substrate. Cells were treated with the ManLev, thereby incorporating a ketone group onto the cell surface. *See,* also Saxon *et al., Science* **287**: 2007 (2000); Hang *et al., J. Am. Chem. Soc.* **123**: 1242 (2001); Yarema *et al., J. Biol. Chem.* **273**: 31168 (1998); and Charter *et al., Glycobiology* **10**: 1049 (2000).

[0021] In addition to an industrially relevant method that utilizes the enzymatic conjugation to specifically conjugate a modified sugar to a peptide or glycopeptide, a method for controlling and manipulating the position of glycosylation on a glycopeptide would be highly desirable.

[0022] Carbohydrates are attached to glycopeptides in several ways of which N-linked to asparagine and mucin-type O-linked to serine and threonine are the most relevant for recombinant glycoprotein therapeuctics. A determining factor for initiation of glycosylation of a protein is the primary sequence context, although clearly other factors including protein

region and conformation play roles. N-linked glycosylation occurs at the consensus sequence NXS/T, where X can be any amino acid but proline.

[0023] O-linked glycosylation is initiated by a family of about 20 homologous enzymes termed UDP-GalNAc: polypeptide *N*-acetylgalactosaminyltransferases (GalNAc-transferases). O-linked glycosylation does not appear to be ruled by one simple consensus sequence, although studies of the GalNAc-transferase enzymes that initiate O-linked glycosylation clearly supports the notion that their acceptor specificities are driven by primary sequence contexts. Each of these enzymes transfer a single monosaccharide GalNAc to serine and threonine residues, but they transfer to different peptide sequences although they show a large degree of overlap in functions. It is envisioned that the substrate specificity of each GalNAc-transferase is ruled primarily by a linear short acceptor consensus sequence.

[0024] Recently, a method of producing an ester linked carbohydrate-peptide conjugate was described by Davis (WO 03/014371, published Feb. 20, 2003). In this publication, a vinyl ester amino acid group was reacted with a carbohydrate acyl acceptor in the presence of an enzyme such as a protease (such as a serine protease), lipase, esterase or acylase. At this time, however, no other substrates, e.g., glycopeptides, glycolipids, are known to conjugate with carbohydrate acyl acceptors under these conditions.

[0025] The present invention answers the need for modified therapeutic species in which a modified glycosyl moiety is conjugated onto N- or O-linked glycosylation sites of the peptides and other bioactive species, *e.g.*, glycolipids, sphingosines, ceramides, etc. The invention provides a route to new therapeutic conjugates and addresses the need for more stable and therapeutically effective therapeutic species. Moreover, despite the efforts directed toward the enzymatic elaboration of saccharide structures, there remains still a need for alternative industrially practical methods for the modification of therapeutic agents, e.g., peptides, glycopeptides and lipids with modifying groups such as water-soluble polymers, therapeutic moieties, biomolecules and the like. Of particular interest are methods in which the modified peptide has improved properties, which enhance its use as a therapeutic or diagnostic agent. The present invention fulfills these and other needs.

BRIEF SUMMARY OF THE INVENTION

[0026] Glycotherapeutics (e.g., glycopeptides, and glycolipids) present a challenging target for recombinant production of therapeutics. For example, carbohydrates are often

indispensable for the function and favorable pharmacokinetic properties of glycopeptide therapeutics; however, many of the most robust expressions systems produce glycopeptides with non-human glycosylation patterns. Incorrect glycosylation can produce a peptide that is inactive, aggregated, antigenic and/or has unfavorable pharmacokinetics. Accordingly, considerable efforts are expended to develop recombinant expression cell systems capable of producing glycoproteins with biologically appropriate carbohydrate structures. This approach is hampered by numerous shortcomings, including cost, and heterogeneity and limitations in glycan structures.

[0027] Post-expression, *in vitro* glyco-modification of glycotherapeutics, e.g., glycopeptides, is an attractive strategy to remedy the deficiencies of methods that rely on controlling glycosylation by engineering expression systems; including both modification of glycan structures or introduction of glycans at novel sites. A comprehensive toolbox of recombinant eukaryotic glycosyltransferases is becoming available, making *in vitro* enzymatic synthesis of glycoconjugates with custom designed glycosylation patterns and glycosyl structures possible. See, for example, U.S. Patent No.s 5,876,980; 6,030,815; 5,728,554; and 5,922,577; and WO 98/31826; US03/180835; and WO 03/031464.

[0028] *In vitro* glycosylation offers a number of advantages compared to recombinant expression of glycoproteins of which custom design and higher degree of homogeneity of the glycosyl moiety are examples. Moreover, combining bacterial expression of glycotherapeutics with *in vitro* modification (or placement) of the glycosyl residue offers numerous advantages over traditional recombinant expression technology including reduced potential exposure to adventitious agents, increased homogeneity of product, and cost reduction.

[0029] Ideally, conjugates of therapeutic species, such as peptides and lipids, are obtained using methods that provide the conjugates in a reproducible and predictable manner. Moreover, in forming the conjugates it is generally preferred that the site of conjugation between the therapeutic species and the modifying group is selected such that its modification does not adversely affect advantageous properties of the therapeutic species, e.g. activity, specificity, low antigenicity, low toxicity, etc.

[0030] The present invention provides a method of forming conjugates between a glycosyl residue, amino acid or aglycone moiety of a selected substrate (e.g., (glyco)peptide, (glyco)lipid, etc.) and a modifying group, such as a water-soluble- or water-insoluble-

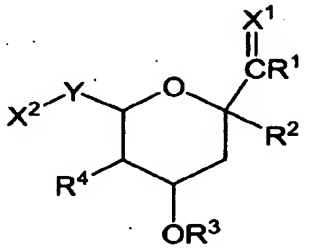
polymer, a therapeutic moiety or a diagnostic agent. The invention exploits the recognition that saccharides, e.g., sialic acid, can be oxidized in a predictable and reproducible fashion by oxidation, converting a primary or secondary hydroxyl moiety to an aldehyde or a ketone. The carbonyl moiety is readily modified with an amine-containing modifying group, affording a Schiff base, which is reduced to the corresponding amine modified saccharyl fragment. The fragment is recognized as a substrate by one or more enzyme capable of transferring a glycosyl moiety onto a substrate.

[0031] In an exemplary embodiment, the modified saccharyl fragment is a substrate for an enzyme that transfers a glycosyl donor moiety to a glycosyl acceptor. In an exemplary embodiment, the enzyme is a transferase, e.g., a sialyltransferase, which utilizes the modified fragment as a saccharyl donor in an enzymatically-mediated glycosylation reaction. In another embodiment, the enzyme is a mutant of a degradative enzyme, such as an exo- or endoglycosidase, amidase, etc.

[0032] In another embodiment, the modified saccharyl fragment is couple to an intact saccharide residue. For example, coupling Sia*-(modifying group) to galactose affords, Gal-Sia*-(modifying group), which serves as a glycosyl donor that is added to a substrate, e.g, peptide, lipid, aglycone, etc.

[0033] The present invention is exemplified by reference to modified saccharyl fragments in which the side chain of a sialic acid is oxidized and the resulting carbonyl moiety is converted to an amine by reductive amination with an amine-containing modifying group. Those of skill will appreciate that saccharides, as a group, possess a rich oxidation chemistry that is readily exploited in variations on the exemplification of the invention presented herein.

[0034] In an exemplary aspect, the present invention provides a conjugate of a bioactive species, e.g., a peptide or a lipid (e.g., ceramide or sphingosine) that includes a subunit according to Formula I:



(I).

[0035] In Formula I, the symbol X^1 represents substituted or unsubstituted alkyl, O or NR^8. R^8 is a member selected from H, OH, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl. Appropriate R^1 groups are selected from OR^9, NR^9R^{10}, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl. The symbols R^9 and R^{10} independently represent H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and $C(O)R^{11}$. R^{11} is a group such as substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl.

[0036] The symbol R^2 is a member selected from an amino acid residue of a peptide, a carbohydrate moiety attached to an amino acid residue of a peptide, or a carbohydrate moiety attached to an amino acid residue of a peptide through a linker. Exemplary linkers include one or more additional carbohydrate moieties in addition to that of R^2. The symbol R^4 represents H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, OH and $NHC(O)R^{12}$. R^{12} is selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl and $NR^{13}R^{14}$, in which R^{13} and R^{14} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl.

[0037] Y is the residue of the sialic acid side chain remaining following oxidation to a carbonyl and subsequent reaction of the carbonyl moiety with a nucleophilic group. Exemplary groups for Y include CH_2, $CH(OH)CH_2$, $CH(OH)CH(OH)CH_2$ when the oxidation leads to formation of an aldehyde that is subsequently reductively aminated. When the aldehyde is converted to an imine species, or the carbonyl is reacted with a phosphorus ylide, Y is typically C, CH(OH)C or CH(OH)CH(OH)C. When the aldehyde is reacted with a Grignard or lithium reagent, exemplary Y groups include C(OH), CH(OH)CH(OH), CH(OH)CH(OH)C(OH) or an elimination product thereof, e.g., dehydration product.

[0038] The symbol X^2 represents groups formed by addition to the carbonyl moiety of the fragment. Exemplary identities for X^2 include substituted or unsubstituted alkyl (e.g., formed by Wittig, Grignard or other appropriate chemistries), and nitrogen-containing species, e.g., NR^6R^7 or $R^6R^7N\text{-}N=$. R^6 and R^7 are independently H, or $C(O)R^{6a}$. The symbols R^6, R^7 and R^{6a} independently represent H, or a modifying group, e.g., biomolecule, therapeutic moiety,

diagnostic moiety, and a water-soluble or water-insoluble polymer. Exemplary saccharyl fragments of the invention include at least one R^6, R^7 and R^{6a} group that is other than H. For example, selected compounds of the invention include one or more R^6, R^7 or R^{6a} moiety that includes a water-soluble polymer moiety within its structure as discussed in detail hereinbelow.

[0039] When X^2 is substituted or unsubstituted alkyl, e.g., an alkene species formed by a Wittig reaction, or saturated species formed by Grignard or lithium chemistries, X^2 can be a water-soluble or –insoluble polymer or other modifying group described herein, which is attached to Y through a linker moiety that includes a substituted or unsubstituted alkyl or heteroalkyl group.

[0040] In another aspect, the invention provides an activated modified saccharyl moiety that is of use in the methods of the invention. In an exemplary embodiment, according to this aspect, the saccharyl fragment has a structure according to Formula I in which R^2 is a nucleotide, forming a nucleotide sugar in which the sugar moiety is, or includes, the saccharyl fragment. R^2 can also be a leaving group (activating group), such as a halogen, sulfonate ester and the like.

[0041] In a third aspect, the invention provides a peptide or lipid conjugate having a population of water-soluble polymer moieties covalently bound thereto through a glycosyl linking group that includes a moiety according to Formula I. In the conjugate of the invention, essentially each member of the population is bound via a glycosyl linking group, that includes a subunit according to Formula I, to an amino acid or glycosyl residue of the peptide, and each amino acid or glycosyl residue to which the linking group is bound has the same structure.

[0042] In a fourth aspect, the invention provides a method of forming a covalent conjugate between a polymer, e.g., water-soluble polymer, and saccharyl acceptor that is a glycosylated-peptide or -lipid, or a non-glycosylated-peptide or -lipid. The polymer is conjugated to the acceptor via a glycosyl linking group that includes a moiety according to Formula I. The glycosyl linking group is interposed between, and covalently linked either directly or indirectly to both the acceptor and the polymer. The method includes contacting the acceptor with a mixture containing a modified saccharyl fragment, generally activated as the nucleotide derivative, and an enzyme for which the modified saccharyl fragment is a substrate. The mixture also includes an enzyme that transfers a saccharyl residue, for which

the modified saccharyl fragment is a substrate. The reaction is conducted under conditions appropriate to form the conjugate. See, for example WO03/031464 and related U.S. and PCT applications.

[0043] In a fifth aspect, the invention provides a conjugate analogous to those described above, in which the modified saccharyl fragment is derivatized with a therapeutic or diagnostic moiety. In an exemplary embodiment, the modifying group is a biomolecule, which can be a therapeutic or diagnostic agent.

[0044] In a further aspect, the present invention provides a composition for forming a conjugate between a peptide or lipid and a modified saccharyl fragment. The composition generally includes an activated analogue of the saccharyl fragment set forth in Formula I, an enzyme for which the saccharyl fragment is a substrate, and a (glyco)peptide or (glyco)lipid acceptor substrate. The modified saccharyl fragment has covalently attached thereto a member selected from water-soluble polymers, therapeutic moieties and biomolecules.

[0045] Also provided is a pharmaceutical composition. The composition includes a pharmaceutically acceptable carrier and a conjugate of the invention in admixture with a pharmaceutically acceptable carrier.

[0046] Other objects and advantages of the invention will be apparent to those of skill in the art from the detailed description that follows.

[0047] **FIG. 1** is a table of sialyltransferases of use in practicing the present invention.

DETAILED DESCRIPTION OF THE INVENTION AND THE PREFERRED EMBODIMENTS

Abbreviations

[0048] Branched or un-branched PEG, poly(ethyleneglycol), including m-PEG, methoxy-poly(ethylene glycol); branched or unbranched PPG, poly(propyleneglycol); m-PPG, methoxy-poly(propylene glycol); Fuc, fucosyl; Gal, galactosyl; GalNAc, N-acetylgalactosaminyl; Glc, glucosyl; GlcNAc, N-acetylglucosaminyl; Man, mannosyl; ManAc, mannosaminyl acetate; Sia, sialic acid; NeuAc, N-acetylneuraminyl; and SA*, sialic acid fragment.

Definitions

[0049] Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry and nucleic acid chemistry and hybridization are those well known and commonly employed in the art. Standard techniques are used for nucleic acid and peptide synthesis. The techniques and procedures are generally performed according to conventional methods in the art and various general references (*see generally*, Sambrook *et al.* MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference), which are provided throughout this document. The nomenclature used herein and the laboratory procedures in analytical chemistry, and organic synthetic described below are those well known and commonly employed in the art. Standard techniques, or modifications thereof, are used for chemical syntheses and chemical analyses.

[0050] The term "alkyl," by itself or as part of another substituent, means, unless otherwise stated, a straight or branched chain, or cyclic hydrocarbon radical, or combination thereof, which may be fully saturated, mono- or polyunsaturated and can include di- and multivalent radicals, having the number of carbon atoms designated (*i.e.* C_1-C_{10} means one to ten carbons). Examples of saturated hydrocarbon radicals include, but are not limited to, groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, isobutyl, sec-butyl, cyclohexyl, (cyclohexyl)methyl, cyclopropylmethyl, homologs and isomers of, for example, n-pentyl, n-hexyl, n-heptyl, n-octyl, and the like. An unsaturated alkyl group is one having one or more double bonds or triple bonds. Examples of unsaturated alkyl groups include, but are not limited to, vinyl, 2-propenyl, crotyl, 2-isopentenyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl), ethynyl, 1- and 3-propynyl, 3-butynyl, and the higher homologs and isomers. The term "alkyl," unless otherwise noted, is also meant to include those derivatives of alkyl defined in more detail below, such as "heteroalkyl." Alkyl groups, which are limited to hydrocarbon groups are termed "homoalkyl".

[0051] The term "alkylene" by itself or as part of another substituent means a divalent radical derived from an alkane, as exemplified, but not limited, by $-CH_2CH_2CH_2CH_2-$, and further includes those groups described below as "heteroalkylene." Typically, an alkyl (or alkylene) group will have from 1 to 24 carbon atoms, with those groups having 10 or fewer

carbon atoms being preferred in the present invention. A "lower alkyl" or "lower alkylene" is a shorter chain alkyl or alkylene group, generally having eight or fewer carbon atoms.

[0052] The terms "alkoxy," "alkylamino" and "alkylthio" (or thioalkoxy) are used in their conventional sense, and refer to those alkyl groups attached to the remainder of the molecule via an oxygen atom, an amino group, or a sulfur atom, respectively.

[0053] The term "heteroalkyl," by itself or in combination with another term, means, unless otherwise stated, a stable straight or branched chain, or cyclic hydrocarbon radical, or combinations thereof, consisting of the stated number of carbon atoms and at least one heteroatom selected from the group consisting of O, N, Si and S, and wherein the nitrogen and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. The heteroatom(s) O, N and S and Si may be placed at any interior position of the heteroalkyl group or at the position at which the alkyl group is attached to the remainder of the molecule. Examples include, but are not limited to, $-CH_2-CH_2-O-CH_3$, $-CH_2-CH_2-NH-CH_3$, $-CH_2-CH_2-N(CH_3)-CH_3$, $-CH_2-S-CH_2-CH_3$, $-CH_2-CH_2,-S(O)-CH_3$, $-CH_2-CH_2-S(O)_2-CH_3$, $-CH=CH-O-CH_3$, $-Si(CH_3)_3$, $-CH_2-CH=N-OCH_3$, and $-CH=CH-N(CH_3)-CH_3$. Up to two heteroatoms may be consecutive, such as, for example, $-CH_2-NH-OCH_3$ and $-CH_2-O-Si(CH_3)_3$. Similarly, the term "heteroalkylene" by itself or as part of another substituent means a divalent radical derived from heteroalkyl, as exemplified, but not limited by, $-CH_2-CH_2-S-CH_2-CH_2-$ and $-CH_2-S-CH_2-CH_2-NH-CH_2-$. For heteroalkylene groups, heteroatoms can also occupy either or both of the chain termini (*e.g.*, alkyleneoxy, alkylenedioxy, alkyleneamino, alkylenediamino, and the like). Still further, for alkylene and heteroalkylene linking groups, no orientation of the linking group is implied by the direction in which the formula of the linking group is written. For example, the formula $-C(O)_2R'-$ represents both $-C(O)_2R'-$ and $-R'C(O)_2-$.

[0054] In general, an "acyl substituent" is also selected from the group set forth above. As used herein, the term "acyl subsituent" refers to groups attached to, and fulfilling the valence of a carbonyl carbon that is either directly or indirectly attached to the polycyclic nucleus of the compounds of the present invention.

[0055] The terms "cycloalkyl" and "heterocycloalkyl", by themselves or in combination with other terms, represent, unless otherwise stated, cyclic versions of "alkyl" and "heteroalkyl", respectively. Additionally, for heterocycloalkyl, a heteroatom can occupy the position at which the heterocycle is attached to the remainder of the molecule. Examples of

cycloalkyl include, but are not limited to, cyclopentyl, cyclohexyl, 1-cyclohexenyl, 3-cyclohexenyl, cycloheptyl, and the like. Examples of heterocycloalkyl include, but are not limited to, 1 –(1,2,5,6-tetrahydropyridyl), 1-piperidinyl, 2-piperidinyl, 3-piperidinyl, 4-morpholinyl, 3-morpholinyl, tetrahydrofuran-2-yl, tetrahydrofuran-3-yl, tetrahydrothien-2-yl, tetrahydrothien-3-yl, 1 –piperazinyl, 2-piperazinyl, and the like.

[0056] The terms "halo" or "halogen," by themselves or as part of another substituent, mean, unless otherwise stated, a fluorine, chlorine, bromine, or iodine atom. Additionally, terms such as "haloalkyl," are meant to include monohaloalkyl and polyhaloalkyl. For example, the term "halo(C_1-C_4)alkyl" is mean to include, but not be limited to, trifluoromethyl, 2,2,2-trifluoroethyl, 4-chlorobutyl, 3-bromopropyl, and the like.

[0057] The term "aryl" means, unless otherwise stated, a polyunsaturated, aromatic, hydrocarbon substituent which can be a single ring or multiple rings (preferably from 1 to 3 rings) which are fused together or linked covalently. The term "heteroaryl" refers to aryl groups (or rings) that contain from one to four heteroatoms selected from N, O, and S, wherein the nitrogen and sulfur atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. A heteroaryl group can be attached to the remainder of the molecule through a heteroatom. Non-limiting examples of aryl and heteroaryl groups include phenyl, 1-naphthyl, 2-naphthyl, 4-biphenyl, 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2-imidazolyl, 4-imidazolyl, pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidyl, 4-pyrimidyl, 5-benzothiazolyl, purinyl, 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 5-isoquinolyl, 2-quinoxalinyl, 5-quinoxalinyl, 3-quinolyl, and 6-quinolyl. Substituents for each of the above noted aryl and heteroaryl ring systems are selected from the group of acceptable substituents described below.

[0058] For brevity, the term "aryl" when used in combination with other terms (*e.g.*, aryloxy, arylthioxy, arylalkyl) includes both aryl and heteroaryl rings as defined above. Thus, the term "arylalkyl" is meant to include those radicals in which an aryl group is attached to an alkyl group (*e.g.*, benzyl, phenethyl, pyridylmethyl and the like) including those alkyl groups in which a carbon atom (*e.g.*, a methylene group) has been replaced by, for example, an oxygen atom (*e.g.*, phenoxymethyl, 2-pyridyloxymethyl, 3-(1-naphthyloxy)propyl, and the like).

[0059] Each of the above terms (*e.g.*, "alkyl," "heteroalkyl," "aryl" and "heteroaryl") include both substituted and unsubstituted forms of the indicated radical. Preferred substituents for each type of radical are provided below.

[0060] Substituents for the alkyl, and heteroalkyl radicals (including those groups often referred to as alkylene, alkenyl, heteroalkylene, heteroalkenyl, alkynyl, cycloalkyl, heterocycloalkyl, cycloalkenyl, and heterocycloalkenyl) are generally referred to as "alkyl substituents" and "heteroakyl substituents," respectively, and they can be one or more of a variety of groups selected from, but not limited to: -OR', =O, =NR', =N-OR', -NR'R", -SR', -halogen, -SiR'R"R"', -OC(O)R', -C(O)R', -CO_2R', -CONR'R", -OC(O)NR'R", -NR"C(O)R', -NR'-C(O)NR"R"', -NR"$C(O)_2$R', -NR-C(NR'R"R"')=NR"", -NR-C(NR'R")=NR"', -S(O)R', -$S(O)_2$R', -$S(O)_2$NR'R", -$NRSO_2$R', -CN and $-NO_2$ in a number ranging from zero to (2m'+1), where m' is the total number of carbon atoms in such radical. R', R", R"' and R"" each preferably independently refer to hydrogen, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, e.g., aryl substituted with 1-3 halogens, substituted or unsubstituted alkyl, alkoxy or thioalkoxy groups, or arylalkyl groups. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R", R"' and R"" groups when more than one of these groups is present. When R' and R" are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 5-, 6-, or 7-membered ring. For example, -NR'R" is meant to include, but not be limited to, 1-pyrrolidinyl and 4-morpholinyl. From the above discussion of substituents, one of skill in the art will understand that the term "alkyl" is meant to include groups including carbon atoms bound to groups other than hydrogen groups, such as haloalkyl (*e.g.*, -CF_3 and $-CH_2CF_3$) and acyl (*e.g.*, -$C(O)CH_3$, -$C(O)CF_3$, -$C(O)CH_2OCH_3$, and the like).

[0061] Similar to the substituents described for the alkyl radical, the aryl substituents and heteroaryl substituents are generally referred to as "aryl substituents" and "heteroaryl substituents," respectively and are varied and selected from, for example: halogen, -OR', =O, =NR', =N-OR', -NR'R", -SR', -halogen, -SiR'R"R"', -OC(O)R', -C(O)R', -CO_2R', -CONR'R", -OC(O)NR'R", -NR"C(O)R', -NR'-C(O)NR"R"', -NR"$C(O)_2$R', -NR-C(NR'R")=NR"', -S(O)R', -$S(O)_2$R', -$S(O)_2$NR'R", -$NRSO_2$R', -CN and $-NO_2$, -R', -N_3, -$CH(Ph)_2$, fluoro(C_1-C_4)alkoxy, and fluoro(C_1-C_4)alkyl, in a number ranging from zero to the total number of open valences on the aromatic ring system; and where R', R", R"' and R"" are preferably independently selected from hydrogen, (C_1-C_8)alkyl and heteroalkyl,

unsubstituted aryl and heteroaryl, (unsubstituted aryl)-(C_1-C_4)alkyl, and (unsubstituted aryl)oxy-(C_1-C_4)alkyl. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R", R'" and R'"" groups when more than one of these groups is present.

[0062] Two of the aryl substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula –T-C(O)-$(CRR')_q$-U-, wherein T and U are independently –NR-, -O-, -CRR'- or a single bond, and q is an integer of from 0 to 3. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula –A-$(CH_2)_r$-B-, wherein A and B are independently –CRR'-, -O-, -NR-, -S-, -S(O)-, -$S(O)_2$-, -$S(O)_2$NR'- or a single bond, and r is an integer of from 1 to 4. One of the single bonds of the new ring so formed may optionally be replaced with a double bond. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula –$(CRR')_s$-X-$(CR"R'")_d$-, where s and d are independently integers of from 0 to 3, and X is –O-, -NR'-, -S-, -S(O)-, -$S(O)_2$-, or –$S(O)_2$NR'-. The substituents R, R', R" and R'" are preferably independently selected from hydrogen or substituted or unsubstituted (C_1-C_6)alkyl.

[0063] As used herein, the term "heteroatom" includes oxygen (O), nitrogen (N), sulfur (S) and silicon (Si).

[0064] The term "nucleic acid" or "polynucleotide" refers to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* **19**:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* **260**:2605-2608 (1985); and Rossolini *et al.*, *Mol. Cell. Probes* **8**:91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene.

[0065] The term "gene" means the segment of DNA involved in producing a polypeptide chain. It may include regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

[0066] The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. "Amino acid mimetics" refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but functioning in a manner similar to a naturally occurring amino acid.

[0067] Amino acids may be referred to herein by either the commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0068] "Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, "conservatively modified variants" refers to those nucleic acids that encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein that encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule.

Accordingly, each silent variation of a nucleic acid that encodes a polypeptide is implicit in each described sequence.

[0069] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

[0070] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

[0071] The following eight groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Glycine (G);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);
7) Serine (S), Threonine (T); and
8) Cysteine (C), Methionine (M)

(*see, e.g.*, Creighton, *Proteins* (1984)).

[0072] Amino acids may be referred to herein by either the common three-letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature

Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0073] The term "mutating" or "mutation," as used in the context of altering the structure or enzymatic activity of a wild-type enzyme, refers to the deletion, insertion, or substitution of any nucleotide or amino acid residue, by chemical, enzymatic, or any other means, in a polynucleotide sequence encoding a that enzyme or the amino acid sequence of a wild-type enzyme, respectively, such that the amino acid sequence of the resulting enzyme is altered at one or more amino acid residues. The site for such an activity-altering mutation may be located anywhere in the enzyme, but is preferably within the active site of the enzyme.

[0074] "Peptide" refers to a polymer in which the monomers are amino acids and are joined together through amide bonds, alternatively referred to as a polypeptide. Additionally, unnatural amino acids, for example, β-alanine, phenylglycine and homoarginine are also included. Amino acids that are not gene-encoded may also be used in the present invention. Furthermore, amino acids that have been modified to include reactive groups, glycosylation sites, polymers, therapeutic moieties, biomolecules and the like may also be used in the invention. All of the amino acids used in the present invention may be either the D - or L - isomer. The L -isomer is generally preferred. In addition, other peptidomimetics are also useful in the present invention. As used herein, "peptide" refers to both glycosylated and unglycosylated peptides. Also included are petides that are incompletely glycosylated by a system that expresses the peptide. For a general review, *see,* Spatola, A. F., in CHEMISTRY AND BIOCHEMISTRY OF AMINO ACIDS, PEPTIDES AND PROTEINS, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983).

[0075] The term "peptide conjugate," refers to species of the invention in which a peptide is conjugated with an acyl-containing group that is attached to the peptide through a sugar residue.

[0076] The term "sialic acid" refers to any member of a family of nine-carbon carboxylated sugars. The most common member of the sialic acid family is N-acetyl-neuraminic acid (2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactononulopyranos-1-onic acid (often abbreviated as Neu5Ac, NeuAc, or NANA). A second member of the family is N-glycolyl-neuraminic acid (Neu5Gc or NeuGc), in which the N-acetyl group of NeuAc is hydroxylated. A third sialic acid family member is 2-keto-3-deoxy-nonulosonic acid (KDN) (Nadano *et al.* (1986) *J. Biol. Chem.* **261**: 11550-11557; Kanamori *et al., J. Biol. Chem.* **265**: 21811-21819 (1990)). Also included are 9-substituted sialic acids such as a 9-O-C_1-C_6 acyl-Neu5Ac like

9-O-lactyl-Neu5Ac or 9-O-acetyl-Neu5Ac, 9-deoxy-9-fluoro-Neu5Ac and 9-azido-9-deoxy-Neu5Ac. For review of the sialic acid family, *see, e.g.*, Varki, *Glycobiology* **2**: 25-40 (1992); *Sialic Acids: Chemistry, Metabolism and Function*, R. Schauer, Ed. (Springer-Verlag, New York (1992)). The synthesis and use of sialic acid compounds in a sialylation procedure is disclosed in international application WO 92/16640, published October 1, 1992.

[0077] As used herein, the term "modified saccharyl fragment," refers to a fragment of a naturally- or non-naturally-occurring carbohydrate that has been modified, typically oxidatively to create a locus for attaching a modifying group. In an exemplary embodiment, the saccharyl fragment is a sialic acid fragment in which the side chain is altered by oxidative degradation. The oxidation produces a carbonyl moiety that is subsequently reductively aminated with an amine analogue of the modifying group. In another exemplary embodiment, the ring structure of the saccharide is linearized by reductive conversion to an alditol (e.g., mannose to mannitol) and derivatized, e.g., at one or more of the primary hydroxyl moieties. Useful, modifying groups include, but are not limited to, water-soluble polymers, water-insoluble polymers, therapeutic moieties, diagnostic moieties, biomolecules and the like.

[0078] The term "water-soluble" refers to moieties that have a detectable degree of solubility in water. Methods to detect and/or quantify water solubility are well known in the art. Exemplary water-soluble polymers include peptides, saccharides, poly(ethers), poly(amines), poly(carboxylic acids) and the like. Peptides can have mixed sequences of be composed of a single amino acid, *e.g.*, poly(lysine), poly(aspartic acid), and poly(glutamic acid). An exemplary polysaccharide is poly(sialic acid). An exemplary poly(ether) is poly(ethylene glycol), e.g., m-PEG. Poly(ethylene imine) is an exemplary polyamine, and poly(acrylic) acid is a representative poly(carboxylic acid).

[0079] The polymer backbone of the water-soluble polymer can be poly(ethylene glycol) (PEG), e.g., m-PEG. However, it should be understood that other related polymers are also suitable for use in the practice of this invention and that the use of the term PEG or poly(ethylene glycol) is intended to be inclusive and not exclusive in this respect. The term PEG includes poly(ethylene glycol) in any of its forms, including alkoxy PEG, alkyl PEG (e.g., mPEG), difunctional PEG, multiarmed PEG, forked PEG, branched PEG, pendent PEG (i.e. PEG or related polymers having one or more functional groups pendent to the polymer backbone), or PEG with degradable linkages therein.

[0080] The polymer backbone can be linear or branched. Branched polymer backbones are generally known in the art. Typically, a branched polymer has a central branch core moiety and a plurality of linear polymer chains linked to the central branch core. PEG is commonly used in branched forms that can be prepared by addition of ethylene oxide to various polyols, such as glycerol, pentaerythritol and sorbitol. The central branch moiety can also be derived from several amino acids, such as lysine. The branched poly(ethylene glycol) can be represented in general form as $R(\text{-PEG-OH})_m$ in which R represents the core moiety, such as glycerol, pentaerythritol, amino acid (e.g., cysteine, serine, di-lysine, tri-lysine, etc.) and *m* represents the number of arms. Multi-armed PEG molecules, such as those described in U.S. Pat. No.s 5,932,462; 5,643,575; European Patent Application 0473,084 A2; WO 96/41813 (and its priority documents), can also be used as the polymer backbone.

[0081] Many other polymers are also suitable for the invention. Polymer backbones that are non-peptidic and water-soluble, with from 2 to about 300 termini, are particularly useful in the invention. Examples of suitable polymers include, but are not limited to, other poly(alkylene glycols), such as poly(propylene glycol) ("PPG"), copolymers of ethylene glycol and propylene glycol and the like, poly(oxyethylated polyol), poly(olefinic alcohol), poly(vinylpyrrolidone), poly(hydroxypropylmethacrylamide), poly(α-hydroxy acid), poly(vinyl alcohol), polyphosphazene, polyoxazoline, poly(N-acryloylmorpholine), such as described in U.S. Pat. No. 5,629,384, which is incorporated by reference herein in its entirety, and copolymers, terpolymers, and mixtures thereof. Although the molecular weight of each chain of the polymer backbone can vary, it is typically in the range of from about 100 Da to about 100,000 Da, often from about 6,000 Da to about 80,000 Da.

[0082] The terms "large-scale" and "industrial-scale" are used interchangeably and refer to a reaction cycle that produces at least about 250 mg, preferably at least about 500 mg, and more preferably at least about 1 gram of glycoconjugate at the completion of a single reaction cycle.

[0083] The term, "glycosyl linking group," as used herein refers to a glycosyl residue that is a fragment of a parent saccharide, generally prepared by oxidation of one or more primary or secondary hydroxyl moieties on the parent saccharide. An exemplary glycosyl linking group is set forth in Formula I, below. As shown in Formula I, the glycosyl linking group covalently joins the modifying group (e.g., PEG moiety, therapeutic moiety, biomolecule) to the molecule to which it is attached. In the methods of the invention, the "glycosyl linking group" is formed by the covalent modification, via an enzymatic glycosylation reaction

linking the agent to an amino acid and/or glycosyl residue on the peptide. The glycosyl linking group can be a saccharide-derived structure that is degraded or degraded and modified prior to the addition of the modifying group (e.g., oxidation→Schiff base formation→reduction). Alternatively, a portion of the glycosyl linking group may be intact. For example, when the glycosyl linking group is Gal-SA* (SA* is the saccharyl fragment), with Gal attached to a peptide or lipid, the Gal can be intact. The glycosyl linking groups of the invention may be derived from a saccharide by addition of glycosyl unit(s) or removal of one or more glycosyl unit from a parent saccharide structure, followed by coupling a saccharyl fragment of the invention to the newly placed or exposed glycosyl residue.

[0084] The term "targeting moiety," as used herein, refers to species that selectively localize in a particular tissue or region of the body. The localization is mediated by specific recognition of molecular determinants, molecular size of the targeting agent or conjugate, ionic interactions, hydrophobic interactions and the like. Other mechanisms of targeting an agent to a particular tissue or region are known to those of skill in the art. Exemplary targeting moieties include antibodies, antibody fragments, transferrin, HS-glycoprotein, coagulation factors, serum proteins, β-glycoprotein, G-CSF, GM-CSF, M-CSF, EPO and the like.

[0085] As used herein, "therapeutic moiety" means any agent useful for therapy including, but not limited to, antibiotics, anti-inflammatory agents, anti-tumor drugs, cytotoxins, and radioactive agents. "Therapeutic moiety" includes prodrugs of bioactive agents, constructs in which more than one therapeutic moiety is bound to a carrier, e.g, multivalent agents. Therapeutic moiety also includes proteins and constructs that include proteins. Exemplary proteins include, but are not limited to, Erythropoietin (EPO), Granulocyte Colony Stimulating Factor (GCSF), Granulocyte Macrophage Colony Stimulating Factor (GMCSF), Interferon (e.g., Interferon-α, -β, -γ), Interleukin (e.g., Interleukin II), serum proteins (e.g., Factors VII, VIIa, VIII, IX, and X), Human Chorionic Gonadotropin (HCG), Follicle Stimulating Hormone (FSH) and Lutenizing Hormone (LH) and antibody fusion proteins (e.g. Tumor Necrosis Factor Receptor ((TNFR)/Fc domain fusion protein)).

[0086] As used herein, "anti-tumor drug" means any agent useful to combat cancer including, but not limited to, cytotoxins and agents such as antimetabolites, alkylating agents, anthracyclines, antibiotics, antimitotic agents, procarbazine, hydroxyurea, asparaginase, corticosteroids, interferons and radioactive agents. Also encompassed within the scope of the term "anti-tumor drug," are conjugates of peptides with anti-tumor activity, *e.g.* TNF-α.

Conjugates include, but are not limited to those formed between a therapeutic protein and a glycoprotein of the invention. A representative conjugate is that formed between PSGL-1 and TNF-α.

[0087] As used herein, "a cytotoxin or cytotoxic agent" means any agent that is detrimental to cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracinedione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Other toxins include, for example, ricin, CC-1065 and analogues, the duocarmycins. Still other toxins include diptheria toxin, and snake venom (*e.g.*, cobra venom).

[0088] As used herein, "a radioactive agent" includes any radioisotope that is effective in diagnosing or destroying a tumor. Examples include, but are not limited to, indium-111, cobalt-60. Additionally, naturally occurring radioactive elements such as uranium, radium, and thorium, which typically represent mixtures of radioisotopes, are suitable examples of a radioactive agent. The metal ions are typically chelated with an organic chelating moiety.

[0089] Many useful chelating groups, crown ethers, cryptands and the like are known in the art and can be incorporated into the compounds of the invention (*e.g.*, EDTA, DTPA, DOTA, NTA, HDTA, *etc.* and their phosphonate analogs such as DTPP, EDTP, HDTP, NTP, *etc*). *See*, for example, Pitt *et al.*, "The Design of Chelating Agents for the Treatment of Iron Overload," In, INORGANIC CHEMISTRY IN BIOLOGY AND MEDICINE; Martell, Ed.; American Chemical Society, Washington, D.C., 1980, pp. 279-312; Lindoy, THE CHEMISTRY OF MACROCYCLIC LIGAND COMPLEXES; Cambridge University Press, Cambridge,1989; Dugas, BIOORGANIC CHEMISTRY; Springer-Verlag, New York, 1989, and references contained therein.

[0090] Additionally, a manifold of routes allowing the attachment of chelating agents, crown ethers and cyclodextrins to other molecules is available to those of skill in the art. *See*, for example, Meares *et al.*, "Properties of In Vivo Chelate-Tagged Proteins and Polypeptides." In, MODIFICATION OF PROTEINS: FOOD, NUTRITIONAL, AND PHARMACOLOGICAL ASPECTS;" Feeney, *et al.*, Eds., American Chemical Society, Washington, D.C., 1982, pp. 370-387; Kasina *et al.*, *Bioconjugate Chem.*, **9**: 108-117 (1998); Song *et al.*, *Bioconjugate Chem.*, **8**: 249-255 (1997).

[0091] As used herein, "pharmaceutically acceptable carrier" includes any material, which when combined with the conjugate retains the conjugates' activity and is non-reactive with the subject's immune systems. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Other carriers may also include sterile solutions, tablets including coated tablets and capsules. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well known conventional methods.

[0092] As used herein, "administering" means oral administration, administration as a suppository, topical contact, intravenous, intraperitoneal, intramuscular, intralesional, or subcutaneous administration, administration by inhalation, or the implantation of a slow-release device, *e.g.*, a mini-osmotic pump, to the subject. Adminsitration is by any route including parenteral and transmucosal (*e.g.*, oral, nasal, vaginal, rectal, or transdermal), particularly by inhalation. Parenteral administration includes, *e.g.*, intravenous, intramuscular, intra-arteriole, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial. Moreover, where injection is to treat a tumor, *e.g.*, induce apoptosis, administration may be directly to the tumor and/or into tissues surrounding the tumor. Other modes of delivery include, but are not limited to, the use of liposomal formulations, intravenous infusion, transdermal patches, etc.

[0093] The term "isolated" refers to a material that is substantially or essentially free from components, which are used to produce the material. For conjugates of the invention, the term "isolated" refers to material that is substantially or essentially free from components, which normally accompany the material in the mixture used to prepare the conjugate. "Isolated" and "pure" are used interchangeably. Typically, isolated conjugates of the invention have a level of purity preferably expressed as a range. The lower end of the range of purity for the conjugates is about 60%, about 70% or about 80% and the upper end of the range of purity is about 70%, about 80%, about 90% or more than about 90%.

[0094] When the conjugates are more than about 90% pure, their purities are also preferably expressed as a range. The lower end of the range of purity is about 90%, about

92%, about 94%, about 96% or about 98%. The upper end of the range of purity is about 92%, about 94%, about 96%, about 98% or about 100% purity.

[0095] Purity is determined by any art-recognized method of analysis (*e.g.*, band intensity on a silver stained gel, polyacrylamide gel electrophoresis, HPLC, or a similar means).

[0096] "Essentially each member of the population," as used herein, describes a characteristic of a population of peptide conjugates of the invention in which a selected percentage of the modified saccharyl fragments added to a peptide are added to multiple, identical acceptor sites on the peptide. "Essentially each member of the population" speaks to the "homogeneity" of the sites on the peptide conjugated to a modified saccharyl fragment and refers to conjugates of the invention, which are at least about 80%, preferably at least about 90% and more preferably at least about 95% homogenous.

[0097] "Homogeneity," refers to the structural consistency across a population of acceptor moieties to which the modified saccharyl fragments are conjugated. Thus, in a peptide conjugate of the invention in which each modified saccharyl fragment moiety is conjugated to a site having the same structure as the site to which every other modified saccharyl fragment is conjugated, the peptide conjugate is said to be about 100% homogeneous. Homogeneity is typically expressed as a range. The lower end of the range of homogeneity for the peptide conjugates is about 60%, about 70% or about 80% and the upper end of the range of purity is about 70%, about 80%, about 90% or more than about 90%.

[0098] When the peptide conjugates are more than or equal to about 90% homogeneous, their homogeneity is also preferably expressed as a range. The lower end of the range of homogeneity is about 90%, about 92%, about 94%, about 96% or about 98%. The upper end of the range of purity is about 92%, about 94%, about 96%, about 98% or about 100% homogeneity. The purity of the peptide conjugates is typically determined by one or more methods known to those of skill in the art, e.g., liquid chromatography-mass spectrometry (LC-MS), matrix assisted laser desorption mass time of flight spectrometry (MALDITOF), capillary electrophoresis, and the like.

[0099] "Substantially uniform conjugate" or a "substantially uniform conjugation pattern," when referring to a glycoconjugate species, refers to the percentage of peptide glycosylation sites that are functionalized directly, or through a glycosyl linker, with a modified saccharyl fragment. A substantially uniform conjugation pattern exists if substantially all (as defined

below) members of a glycosylation site population intended to bear the modified saccharyl fragment are directly or indirectly functionalized with that fragment.

[0100] The term "substantially" in the above definitions of "substantially uniform" generally means at least about 40%, at least about 70%, at least about 80%, or more preferably at least about 90%, and still more preferably at least about 95% of the acceptor moieties for a particular modified saccharyl fragment are modified by that fragment.

[0101] The terms "(glyco)peptide" and "(glyco)lipid," refer, respectively, to peptide and glycopeptide; and lipid and glycolipid. The terms "peptide" and "lipid" are used generically to refer to both glycosylated and non-glycosylated analogues of these species.

Introduction

[0102] The present invention provides conjugates bearing one or more modified saccharyl fragment moiety. The modified fragment is attached to an acceptor moiety on a substrate, e.g., an amino acid or glycosyl residue of a peptide or glycopeptide, or onto an aglycone or glycosyl residue of a glycolipid (*e.g.*, sphingosine, ceramide, etc.). Also provided are enzymatically-mediated methods for producing the conjugates of the invention, and activated modified saccharyl fragments of use in the methods. The invention also provides pharmaceutical formulations that include a conjugate formed by a method of the invention.

[0103] Conjugates of the invention are formed between a therapeutic core molecule, *e.g.*, (glyco)peptide, (glyco)lipid, and diverse modifying groups such as water-soluble polymers, therapeutic moieties, diagnostic moieties, targeting moieties and the like. The modifying group is conjugated to the therapeutic species through a saccharyl fragment. Also provided are conjugates that include two or more peptides linked together through a linker arm, *i.e.*, multifunctional conjugates. The multi-functional conjugates of the invention can include two or more copies of the same peptide or a collection of diverse peptides with different structures and/or properties. In exemplary conjugates according to this embodiment, the linker between the two peptides includes at least one saccharyl fragment, or modified saccharyl fragment as described herein.

[0104] The conjugates of the invention are prepared by the enzymatic conjugation of an activated modified saccharyl fragment to a therapeutic substrate. When the conjugate of the invention is a glycopeptide conjugate, the modified saccharyl fragment is attached directly to an amino acid of a glycosylation site, or to a glycosyl residue attached either directly or indirectly (e.g., through one or more glycosyl residue) to a glycosylation site.

[0105] The invention also provides lipid conjugates in which the modified saccharyl fragment is attached to an aglycone moiety of a lipid or to a glycosyl residue of a glycolipid.

[0106] The modified saccharyl fragment, when interposed between the peptide (or glycosyl residue) and the modifying group, becomes what is referred to herein as a "glycosyl linking group." Using the exquisite selectivity of enzymes, such as glycosyl transferases, amidases, endoglycanases, endoglycoceramidases, and the like, the present method provides peptides and lipids that bear a desired group at one or more specific locations. Thus, in exemplary conjugates according to the present invention, a modified saccharyl fragment is attached directly to a selected locus on the peptide chain or, alternatively, the modified saccharyl fragment is appended onto a carbohydrate moiety of a glycopeptide. Peptides in which modified saccharyl fragments are bound to both a glycopeptide carbohydrate and directly to an amino acid residue of the peptide backbone are also within the scope of the present invention.

[0107] The methods of the invention make it possible to assemble modified glycopeptides and glycolipids that have a substantially homogeneous derivatization pattern; the enzymes used in the invention are generally selective for a particular glycosyl residue or for particular substituents, or substituent patterns, on a glycosyl residue. The methods are also practical for large-scale production of modified glycopeptide and glycolipid conjugates. In one embodiment the methods of the invention provide a practical means for large-scale preparation of glycopeptide and glycolipid conjugates having preselected uniform derivatization patterns. The methods are particularly well suited for modification of therapeutic peptides, including but not limited to, glycopeptides that are incompletely glycosylated during production in cell culture cells (*e.g.*, mammalian cells, insect cells, plant cells, fungal cells, yeast cells, or prokaryotic cells) or transgenic plants or animals.

[0108] The methods of the invention also provide conjugates of glycosylated and unglycosylated peptides, and glycolipids, with increased therapeutic half-life due to, for example, reduced clearance rate, or reduced rate of uptake by the immune or reticuloendothelial system (RES). Moreover, the methods of the invention provide a means for masking antigenic determinants on peptides, thus reducing or eliminating a host immune response against the peptide. Selective attachment of targeting agents to a peptide or glycolipid using an appropriate modified saccharyl fragment can also be used to target the peptide or glycolipid to a particular tissue or cell surface receptor that is specific for the

particular targeting agent. Moreover, there is provided a class of peptides and glycolipids that are specifically modified with a therapeutic moiety conjugated through a glycosyl linking group.

The Embodiments

Compositions: Glyco-conjugates

[0109] The present invention provides glyco-conjugates that include a saccharyl fragment functionalized with a modifying group. When the saccharyl fragment is formed by oxidation of a saccharide, e.g., sialic acid, the reagent used to conjugate the modifying group to the oxidized saccharide fragment generally includes a group that reacts with a carbonyl moiety formed during the oxidation.

Modified saccharyl fragments

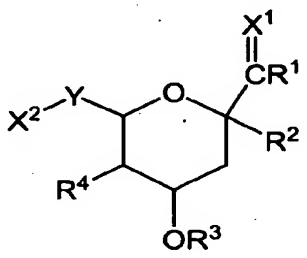
[0110] The present invention provides compounds and methods that are based upon the discovery that enzymes capable of transferring an intact glycosyl moiety to an acceptor substrate are also capable of transferring a modified saccharyl fragment to the acceptor. Accordingly, the invention is not limited by the structure or methods of obtaining appropriate saccharyl fragments or modified saccharyl fragments.

[0111] In an exemplary embodiment, the saccharide fragment is prepared by the oxidative degradation of the parent saccharide. Methods of selectively oxidizing saccharide groups are well known in the art. For example, the periodate ion is of use to cleave vicinal diols, forming the corresponding dialdehyde. Controlled periodate oxidation of the side chain of sialic acid leads to the formation of an oxidized or oxidized and truncated side chain bearing an aldehyde. By chosing appropriate conditions, a side chain containing from one to three carbon atoms is produced. See, for example, Chai et al., *Carbohydr. Res.* **239**: 107-115 (1993); and Murray et al., *Carbohydr. Res.* **186**: 107-115 (1989).

[0112] The carbonyl moiety introduced into the saccharyl fragment undergoes those reactions generally used for the modification of a carbonyl moiety. For example, modifying groups that include amines are of use as are those that form imines, e.g., hydrazines, semicarbazines and the like. Other typical reactions include the reaction of the carbonyl moiety with ylides (e.g., sulfur and phosphorus), and with Grignard and lithium reagents.

[0113] An exemplary modified saccharyl fragment of the invention is formed by the oxidative degradation of the side chain of sialic acid. The oxidation leads to the formation of

a carbonyl moiety that is reductively aminated with an amine derivative of a modifying group of interest. Thus, in this embodiment, the invention provides a modified saccharyl fragment having a structure according to Formula I:



(I).

[0114] In Formula I, the symbol X^1 represents O or NR^8. R^8 is a member selected from H, OH, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl. Appropriate R^1 groups are selected from OR^9, NR^9R^{10}, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl. The symbols R^9 and R^{10} independently represent H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and $C(O)R^{11}$. R^{11} is a group such as substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl.

[0115] The symbol R^2 is a member selected from an amino acid residue of a peptide, a carbohydrate moiety attached to an amino acid residue of a peptide, or a carbohydrate moiety attached to an amino acid residue of a peptide through a linker. Exemplary linkers include one or more additional carbohydrate moieties in addition to that of R^2. R^3 is a member selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl. The symbol R^4 represents H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, OH and $NHC(O)R^{12}$. R^{12} is selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl and $NR^{13}R^{14}$, in which R^{13} and R^{14} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl.

[0116] Y is the residue of the sialic acid side chain remaining following oxidation. Exemplary groups for Y include CH_2, $CH(OH)CH_2$, $CH(OH)CH(OH)CH_2$ when the oxidation leads to formation of an aldehyde that is subsequently reductively aminated, or when the product results from addition of a phosphorus or sulfur ylide. When the aldehyde is

converted to an imine species, Y is typically C, CH(OH)C or CH(OH)CH(OH)C. When the aldehyde is reacted with a Grignard or lithium reagent, exemplary Y groups include C(OH), CH(OH)CH(OH), CH(OH)CH(OH)C(OH) or an elimination product thereof, e.g., dehydration product.

[0117] The symbol X^2 represents groups formed by addition to the carbonyl moiety of the fragment. Exemplary identities for X^2 include substituted or unsubstituted alkyl, and nitrogen-containing species, e.g., NR^6R^7 or R^6R^7N-N=. R^6 and R^7 are independently H, or $C(O)R^{6a}$. The symbols R^6, R^7 and R^{6a} independently represent H, or a modifying group, e.g., biomolecule, therapeutic moiety, diagnostic moiety, and a water-soluble or water-insoluble polymer. Exemplary saccharyl fragments of the invention include at least one R^6, R^7 and R^{6a} group that is other than H. For example, selected compounds of the invention include one or more R^6, R^7 or R^{6a} moiety that includes a water-soluble polymer moiety within its structure as discussed in detail hereinbelow.

[0118] When X^2 is substituted or unsubstituted alkyl, e.g., species formed by Wittig, Grignard or lithium chemistries, X^2 can be a water-soluble or –insoluble polymer or other modifying group described herein, which is attached to Y through a linker moiety that includes a substituted or unsubstituted alkyl or heteroalkyl group.

[0119] In an exemplary embodiment, the modified saccharyl fragment is prepared by reacting a carbonyl-containing sacchary fragment with a Wittig reagent that includes a water-soluble polymer, e.g., m-PEG. Wittig reagents of m-PEG are readily formed by reaction of chloro-m-PEG with PPh_3 and treating the resulting adduct with a base to form the ylide. Other ylides of use in forming the compounds of the invention are prepared by deprotonating an alkyl phosphonate according to the Arbuzov reaction and reacting the saccharyl fragment carbonyl moiety with this ylide under conditions appropriate for the Horner-Emmons reaction.

[0120] Grignard reagents of use in present invention, e.g. m-PEGMgBr, are readily prepared according to art-recognized methods. For example, m-PEG-Br is reacted with Mg under anhydrous conditions.

[0121] In another exemplary embodiment, the carbonyl-containing fragment is reductively aminated with ammonia. The resulting amine is alkylated or acylated with a selected modifying group, e.g., m-PEG or branched m-PEG.

[0122] Typically, the saccharyl fragment is a monosaccharide; however, because the side chain of sialic acid is selectively oxidized in the presence of the vicinal diols of other saccharides, the present invention is not limited to the use of modified sialic acid, but is of use with sialic acid fragment-containing oligosaccharides and polysaccharides as well.

[0123] In another aspect, the invention provides an activated modified saccharyl fragment that is of use in the methods of the invention. An exemplary activated modified saccharyl fragment includes an activated leaving group. As used herein, the term "activated leaving group" refers to those moieties, which are easily displaced in enzyme-regulated nucleophilic substitution reactions. Many activated sugars are known in the art. See, for example, Vocadlo et al., In CARBOHYDRATE CHEMISTRY AND BIOLOGY, Vol. 2, Ernst *et al.* Ed., Wiley-VCH Verlag: Weinheim, Germany, 2000; Kodama *et al., Tetrahedron Lett.* **34**: 6419 (1993); Lougheed, *et al., J. Biol. Chem.* **274**: 37717 (1999)).

[0124] In an exemplary embodiment, according to this aspect, the saccharyl fragment has a structure according to Formula I in which R^2 is an activating group. An exemplary activating group is a nucleotide, forming a nucleotide sugar in which the sugar moiety is the saccharyl fragment. R^2 can also be a leaving group (activating group), such as a halogen, sulfonate ester and the like.

[0125] An exemplary activated leaving group is a nucleotide, which can be utilized to add the modified saccharyl fragment to an acceptor moiety on the substrate. Exemplary sugar nucleotides present in the compounds of the invention include nucleotide mono-, di- or triphosphates or analogs thereof. In a preferred embodiment, the modified saccharyl fragment nucleotide is selected from a UDP-glycoside, CMP-glycoside, or a GDP-glycoside. Even more preferably, the modified saccharyl fragment nucleotide is selected from analogues of UDP-galactose, UDP-galactosamine, UDP-glucose, UDP-glucosamine, GDP-mannose, GDP-fucose, CMP-sialic acid or CMP-NeuAc in which the saccharyl moiety (other than the nucleotide ribose) is a saccharyl fragment bearing a modifying group.

[0126] In an exemplary embodiment, one or more sugar nucleotides or modified sugar nucleotides are used in conjunction with a glycosyltransferase.

[0127] In other embodiments, the activating moiety is an activated leaving group other than a nucleotide. Examples of non-nucleotide activating groups include fluoro, chloro, bromo, tosylate ester, mesylate ester, triflate ester and the like. Preferred activated leaving groups, for use in the present invention, are those that do not significantly sterically encumber the

enzymatic transfer of the glycoside to the acceptor. Accordingly, preferred embodiments of activated glycoside derivatives include glycosyl fluorides and glycosyl mesylates, with glycosyl fluorides being particularly preferred. Among the glycosyl fluorides, α-galactosyl fluoride, α-mannosyl fluoride, α-glucosyl fluoride, α-fucosyl fluoride, α-xylosyl fluoride, α-sialyl fluoride, α-N-acetylglucosaminyl fluoride, α-N-acetylgalactosaminyl fluoride, β-galactosyl fluoride, β-mannosyl fluoride, β-glucosyl fluoride, β-fucosyl fluoride, β-xylosyl fluoride, β-sialyl fluoride, β-N-acetylglucosaminyl fluoride and β-N-acetylgalactosaminyl fluoride are most preferred.

[0128] By way of illustration, glycosyl fluorides can be prepared from the saccharyl fragment or modified saccharyl fragment by first acetylating the sugar and then treating it with HF/pyridine. This generates the thermodynamically most stable anomer of the protected (acetylated) glycosyl fluoride (*i.e.*, the α-glycosyl fluoride). If the less stable anomer (*i.e.*, the β-glycosyl fluoride) is desired, it can be prepared by converting the peracetylated sugar with HBr/HOAc or with HCl to generate the anomeric bromide or chloride. This intermediate is reacted with a fluoride salt such as silver fluoride to generate the glycosyl fluoride. Acetylated glycosyl fluorides may be deprotected by reaction with mild (catalytic) base in methanol (*e.g.* NaOMe/MeOH). In addition, many glycosyl fluorides are commercially available.

[0129] Other activated glycosyl derivatives can be prepared using conventional methods known to those of skill in the art. For example, glycosyl mesylates can be prepared by treatment of the fully benzylated hemiacetal form of the sugar with mesyl chloride, followed by catalytic hydrogenation to remove the benzyl groups.

[0130] In an exemplary embodiment, one or more activated glycosyl derivative such as those set forth above is used in conjunction with an enzyme that is a mutant of a degradative enzyme; mutated to enhance its activity forming glycosidic and amino-glycosidic bonds remutant exo- or endo-glycosidase relative to the activity of the wild-type to cleave these bonds. Enzymes of use in this embodiment include those described in WO03/046150, WO03/045980, and their US counterpart patent applications).

[0131] In addition to including a moiety according to Formula I, the conjugates of the invention can include one or more additional modified saccharyl fragment appended to an amino acid, aglycone or glycosyl residue of the conjugate. The structure and preparation of exemplary modified saccharyl fragments that are of use in combination with the modified

saccharyl fragment of the invention are also disclosed in WO03/031464 and related U.S. and PCT applications.

Sugars

[0132] Any sugar can be utilized as the sugar core of the modified saccharyl fragment conjugates of the invention. Exemplary sugar cores that are useful in forming the compositions of the invention include, but are not limited to, sialic acid, glucose, galactose, and mannose and N-acetyl analogues of these sugars. Also of use are fucose, xylose, ribose, and arabinose. Also encompassed within the invention are species in which the sugar core is a disaccharide, an oligosaccharide or a polysaccharide.

Modifying Groups and Activated Modifying Groups

[0133] Another glyco-conjugate component is the modifying group. As will be apparent from the description that follows, the modifying groups of the invention can be any group, *e.g.*, water-soluble polymer, water-insoluble polymer, therapeutic moiety, etc., that can be conjugated to a saccharyl fragment.

Water-Soluble Polymers

[0134] Many water-soluble polymers are known to those of skill in the art and are useful in practicing the present invention. The term water-soluble polymer encompasses species such as saccharides (e.g., dextran, amylose, hyalouronic acid, poly(sialic acid), heparans, heparins, etc.); poly (amino acids), e.g., poly(aspartic acid) and poly(glutamic acid); nucleic acids; synthetic polymers (e.g., poly(acrylic acid), poly(ethers), e.g., poly(ethylene glycol); peptides, proteins, and the like. The present invention may be practiced with any water-soluble polymer with the sole limitation that the polymer must include a point at which the remainder of the conjugate can be attached.

[0135] Methods for activation of polymers can also be found in WO 94/17039, U.S. Pat. No. 5,324,844, WO 94/18247, WO 94/04193, U.S. Pat. No. 5,219,564, U.S. Pat. No. 5,122,614, WO 90/13540, U.S. Pat. No. 5,281,698, and more WO 93/15189, and for conjugation between activated polymers and peptides, *e.g.* Coagulation Factor VIII (WO 94/15625), hemoglobin (WO 94/09027), oxygen carrying molecule (U.S. Pat. No. 4,412,989), ribonuclease and superoxide dismutase (Veronese *at al., App. Biochem. Biotech.* 11: 141-45 (1985)).

[0136] Exemplary activated water-soluble polymers of the invention include a moiety that is reactive towards the saccharyl fragment, allowing the formation of a covalent bond between the fragment and the modifying group. For example, when the saccharyl fragment includes an aldehyde moiety, the water-soluble polymer can include an amine, or an imine-forming species, e.g., a hydrazine, or a semicarbazine group, that reacts with the aldehyde. When the water-soluble polymer is a branched water-soluble polymer, such as those based upon an amino acid or di- or tri-peptidyl residue, the carboxylic acid moiety of the residue is converted to an amine, or an imine-forming species by methods known in the art.

[0137] Preferred water-soluble polymers are those in which a substantial proportion of the polymer molecules in a sample of the polymer are of approximately the same molecular weight; such polymers are "homodisperse."

[0138] The present invention is further illustrated by reference to a poly(ethylene glycol) conjugate. Several reviews and monographs on the functionalization and conjugation of PEG are available. *See,* for example, Harris, *Macronol. Chem. Phys.* **C25**: 325-373 (1985); Scouten, *Methods in Enzymology* **135**: 30-65 (1987); Wong *et al.*, *Enzyme Microb. Technol.* **14**: 866-874 (1992); Delgado *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems* **9**: 249-304 (1992); Zalipsky, *Bioconjugate Chem.* **6**: 150-165 (1995); and Bhadra, *et al.*, *Pharmazie*, **57**:5-29 (2002). Routes for preparing reactive PEG molecules and forming conjugates using the reactive molecules are known in the art. For example, U.S. Patent No. 5,672,662 discloses a water soluble and isolatable conjugate of an active ester of a polymer acid selected from linear or branched poly(alkylene oxides), poly(oxyethylated polyols), poly(olefinic alcohols), and poly(acrylomorpholine).

[0139] U.S. Patent No. 6,376,604 sets forth a method for preparing a water-soluble 1-benzotriazolylcarbonate ester of a water-soluble and non-peptidic polymer by reacting a terminal hydroxyl of the polymer with di(1-benzotriazoyl)carbonate in an organic solvent. The active ester is used to form conjugates with a biologically active agent such as a protein or peptide.

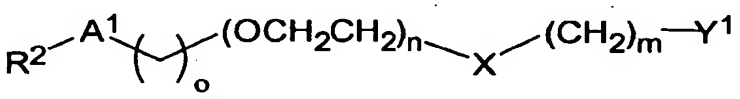
[0140] WO 99/45964 describes a conjugate comprising a biologically active agent and an activated water soluble polymer comprising a polymer backbone having at least one terminus linked to the polymer backbone through a stable linkage, wherein at least one terminus comprises a branching moiety having proximal reactive groups linked to the branching moiety, in which the biologically active agent is linked to at least one of the proximal reactive

groups. Other branched poly(ethylene glycols) are described in WO 96/21469, U.S. Patent No. 5,932,462 describes a conjugate formed with a branched PEG molecule that includes a branched terminus that includes reactive functional groups. The free reactive groups are available to react with a biologically active species, such as a protein or peptide, forming conjugates between the poly(ethylene glycol) and the biologically active species. U.S. Patent No. 5,446,090 describes a bifunctional PEG linker and its use in forming conjugates having a peptide at each of the PEG linker termini.

[0141] Conjugates that include degradable PEG linkages are described in WO 99/34833; and WO 99/14259, as well as in U.S. Patent No. 6,348,558. Such degradable linkages are applicable in the present invention.

[0142] Although both reactive PEG derivatives and conjugates formed using the derivatives are known in the art, until the present invention, it was not recognized that a conjugate could be formed selectively between a specific site on a glycopeptide or glycolipid and PEG (or other polymer) through an intact glycosyl linking group.

[0143] In another exemplary embodiment, poly(ethylene glycol) molecules of the invention include, but are not limited to, those species set forth below.



in which R^2 is H, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted heteroalkyl, e.g., acetal, OHC-, H_2N-$(CH_2)_q$-, HS-$(CH_2)_q$, and-$(CH_2)_qC(Y^3)Z^1$; -sugar-nucleotide, or peptide. The index "n" represents an integer from 1 to 2500. The indeces m, o, and q independently represent integers from 0 to 20. The symbol Z^1 is OH, NH_2, halogen, S-R^{23}, the alcohol portion of activated esters, -$(CH_2)_pC(Y^2)V$, -$(CH_2)_pU(CH_2)_sC(Y^2)_v$, sugar-nucleotide, protein, or leaving groups, e.g., imidazole, p-nitrophenyl, HOBT, tetrazole, halide. The symbols X, Y^2, Y^3, A^1, and U independently represent the moieties O, S, N-R^{24}. Y^1 is OH or other reactive group, or O-alkyl, e.g., O-methyl. The symbol V represents OH, NH_2, halogen, S-R^{25}, the alcohol component of activated esters, the amine component of activated amides, sugar-nucleotides, and proteins. The indeces p, q, s and v are members independently selected from the integers from 0 to 20. The symbols R^{23}, R^{24} and R^{25} independently represent H, substituted or

unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heterocycloalkyl and substituted or unsubstituted heteroaryl.

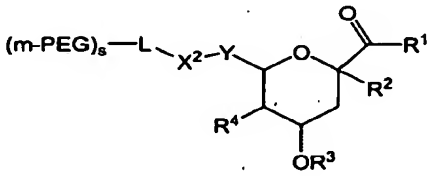
[0144] The poly(ethylene glycol) useful in forming the conjugate of the invention is either linear or branched. In an exemplary embodiment, the modified saccharyl fragment includes the moiety:

$$-L-(m\text{-}PEG)_s$$

wherein L is a bond or a linker moiety, e.g., an amino acid residue or a peptidyl residue; and s is an integer from 1 to 3. Exemplary amino acids are those with side chains that can be functionalized with a water-soluble polymer, e.g., serine, cysteine, lysine, tyrosine, glutamic acid, aspartic acid, etc. When L is a peptidyl residue, it is preferably a di- or tri-peptide that includes one or more sites that can be functionalized with a water-soluble polymer, e.g., di-lysine, tri-lysine, etc.

[0145] An exemplary water-soluble polymer is poly(ethylene glycol), e.g., methoxy-poly(ethylene glycol). The poly(ethylene glycol) used in the present invention is not restricted to any particular form or molecular weight range. For unbranched poly(ethylene glycol) molecules the molecular weight is preferably between 500 and 100,000. A molecular weight of 2,000-60,000 daltons is preferably used and more preferably of from about 5,000 to about 30,000 daltons.

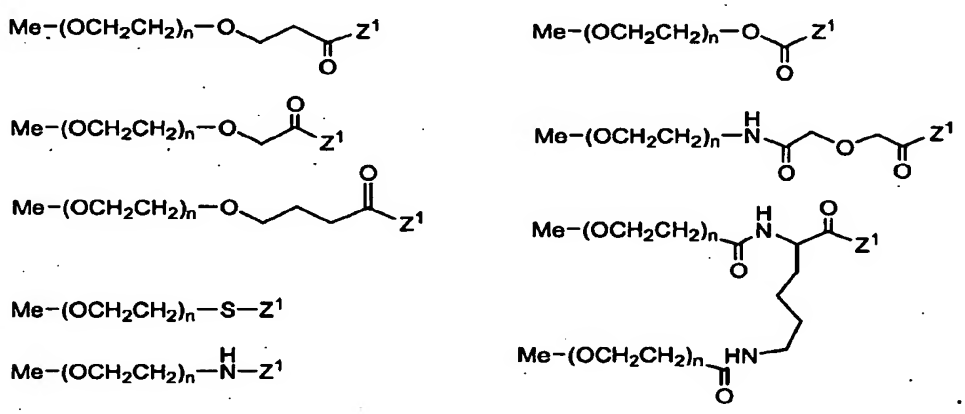
[0146] An exemplary modified fragment according to this motif has the formula:



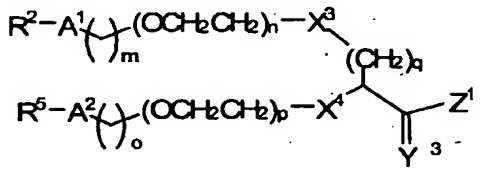
wherein R^2 is OH, NH, an activating group or a bond or linker that joins the fragment to a peptide, lipid or other aglycone or other species of interest. Exemplary activating groups include nucleotides, affording a nucleotide sugar fragment according to Formula I. When a species according to Formula I is incorporated into a conjugate with a peptide, lipid or other aglycone, R^2 is a nucleotide, e.g, CMP; an amino acid, or a carbohydrate moiety attached to an amino acid residue of a peptide, optionally through a linker comprising at least a second carbohydrate moiety. When the fragment is incorporated into a lipid conjugate, R^2 is an

aglycone, or a carbohydrate moiety attached to the aglycone, optionally through a linker that includes at least a one other carbohydrate moiety in addition to the saccharyl fragment of the invention.

[0147] In other exemplary embodiments, the poly(ethylene glycol) molecule or $(m\text{-}PEG)_s$-L moiety is selected from the following:

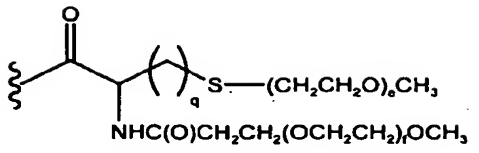


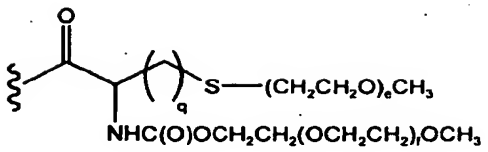
[0148] In general, branched poly(ethylene glycol) molecules suitable for use in the invention include, but are not limited to, those described by the following formula:

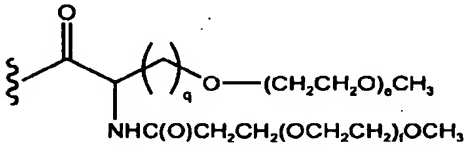


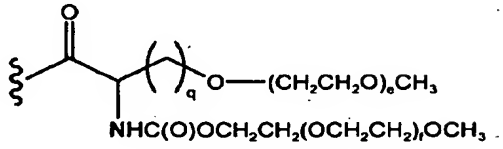
in which R^2 and R^5 are members independently selected from the groups defined for R^2, above. A^1 and A^2 are members independently selected from the groups defined for A^1, above. The indeces m, n, o, p and q are as described above. Z^1 and Y^3 are as described above. X^3 and X^4 are members independently selected from S, SC(O), O, NH, NHC(O) and NHC(O)O.

[0149] In other exemplary embodiments, the branched PEG is based upon a cysteine, serine or small (e.g., 2-10, preferably 3-5), preferably, di-lysine core. Thus, further exemplary branched PEGs include:

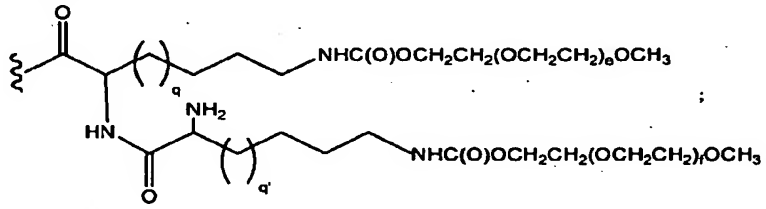


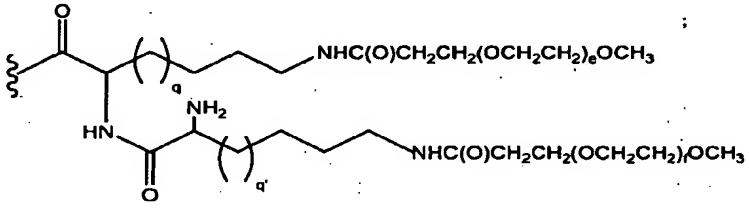
;




; and


wherein e and f are integers independently selected from 1 to 2500; and q is an integer from 1 to 20. In other embodiments R^1 has a structure that is a member selected from:



;


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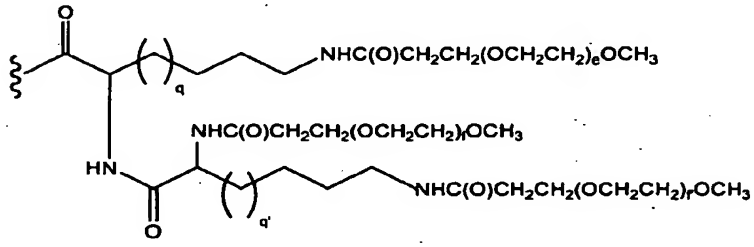
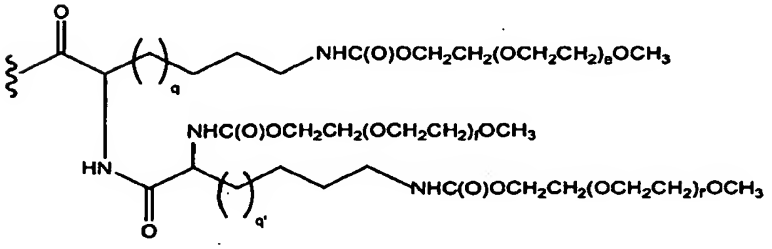
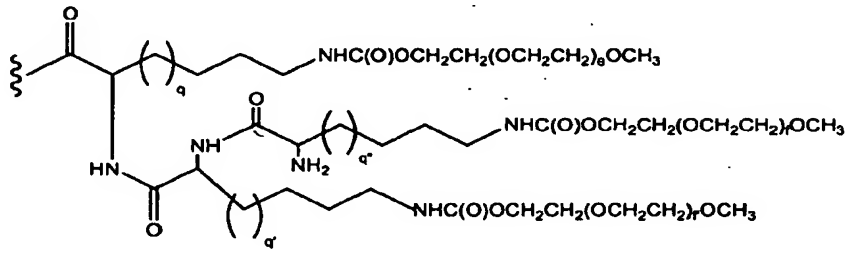


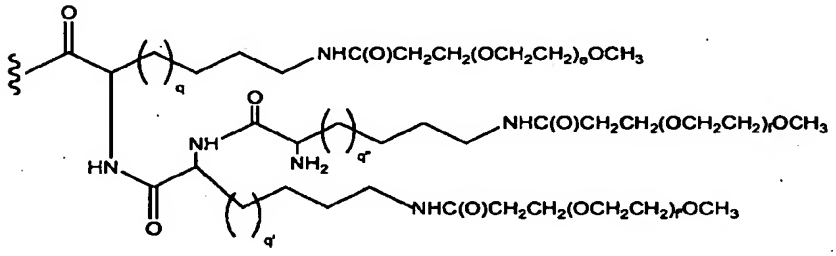
; and


wherein e, f and f' are integers independently selected from 1 to 2500; and q and q' are integers independently selected from 1 to 20.

[0150] In still another embodiment, the invention provides a Factor IX peptide conjugate wherein R^1 has a structure that is a member selected from:

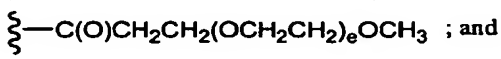


; and

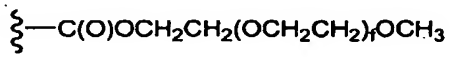


wherein e, f and f' are integers independently selected from 1 to 2500; and q, q' and q"are integers independently selected from 1 to 20.

[0151] In other embodiments, R^1 has a structure that is a member selected from:



; and



wherein e and f are integers independently selected from 1 to 2500.

[0152] As will be apparent to those of skill, the branched polymers of use in the invention include variations on the themes set forth above. For example the di-lysine-PEG conjugate shown above can include three polymeric subunits, the third bonded to the α-amine shown as unmodified in the structure above. Similarly, the use of a tri-lysine functionalized with three or four polymeric subunits is within the scope of the invention.

[0153] Those of skill in the art will appreciate that one or more of the m-PEG arms of the branched polymer can be replaced by a PEG moiety with a different terminus, e.g., OH, COOH, NH_2, C_2-C_{10}-alkyl, etc. Moreover, the structures above are readily modified by

inserting alkyl linkers (or removing carbon atoms) between the α-carbon atom and the functional group of the side chain. Thus, "homo" derivatives and higher homologues, as well as lower homologues are within the scope of cores for branched PEGs of use in the present invention. Furthermore, one or more PEG moiety can be replaced by a modifying group other than a water-soluble polymer, e.g., therapeutic moiety, biomolecule, or water-insoluble polymer.

[0154] In an exemplary embodiment the molecular weight of each poly(ethylene glycol) of the branched PEG is equal to or greater than about 2,000, 5,000, 10,000, 15,000, 20,000, 40,000, 50,000 and 60,000 daltons.

Water-Insoluble Polymers

[0155] In another embodiment, analogous to those discussed above, the modifying group is a water-insoluble polymer, rather than a water-soluble polymer. The glyco-conjugates of the invention may also include one or more water-insoluble polymers. This embodiment of the invention is illustrated by the use of the conjugate as a vehicle with which to deliver a therapeutic peptide in a controlled manner. Polymeric drug delivery systems are known in the art. *See*, for example, Dunn *et al.*, Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991. Those of skill in the art will appreciate that substantially any known drug delivery system is applicable to the conjugates of the present invention.

[0156] Representative water-insoluble polymers include, but are not limited to, polyphosphazines, poly(vinyl alcohols), polyamides, polycarbonates, polyalkylenes, polyacrylamides, polyalkylene glycols, polyalkylene oxides, polyalkylene terephthalates, polyvinyl ethers, polyvinyl esters, polyvinyl halides, polyvinylpyrrolidone, polyglycolides, polysiloxanes, polyurethanes, poly(methyl methacrylate), poly(ethyl methacrylate), poly(butyl methacrylate), poly(isobutyl methacrylate), poly(hexyl methacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate) polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly (ethylene terephthalate), poly(vinyl acetate), polyvinyl chloride, polystyrene, polyvinyl pyrrolidone, pluronics and polyvinylphenol and copolymers thereof.

[0157] Synthetically modified natural polymers of use in the glyco-conjugates of the invention include, but are not limited to, alkyl celluloses, hydroxyalkyl celluloses, cellulose

ethers, cellulose esters, and nitrocelluloses. Particularly preferred members of the broad classes of synthetically modified natural polymers include, but are not limited to, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxymethyl cellulose, cellulose triacetate, cellulose sulfate sodium salt, and polymers of acrylic and methacrylic esters and alginic acid.

[0158] These and the other polymers discussed herein can be readily obtained from commercial sources such as Sigma Chemical Co. (St. Louis, MO.), Polysciences (Warrenton, PA.), Aldrich (Milwaukee, WI.), Fluka (Ronkonkoma, NY), and BioRad (Richmond, CA), or else synthesized from monomers obtained from these suppliers using standard techniques.

[0159] Representative biodegradable polymers of use in the conjugates of the invention include, but are not limited to, polylactides, polyglycolides and copolymers thereof, poly(ethylene terephthalate), poly(butyric acid), poly(valeric acid), poly(lactide-co-caprolactone), poly(lactide-co-glycolide), polyanhydrides, polyorthoesters, blends and copolymers thereof. Of particular use are compositions that form gels, such as those including collagen, pluronics and the like.

[0160] The polymers of use in the invention include "hybrid' polymers that include water-insoluble materials having within at least a portion of their structure, a bioresorbable molecule. An example of such a polymer is one that includes a water-insoluble copolymer, which has a bioresorbable region, a hydrophilic region and a plurality of crosslinkable functional groups per polymer chain.

[0161] For purposes of the present invention, "water-insoluble materials" includes materials that are substantially insoluble in water or water-containing environments. Thus, although certain regions or segments of the copolymer may be hydrophilic or even water-soluble, the polymer molecule, as a whole, is not substantially soluble in water.

[0162] For purposes of the present invention, the term "bioresorbable molecule" includes a region that is capable of being metabolized or broken down and resorbed and/or eliminated through normal excretory routes by the body. Such metabolites or break down products are preferably substantially non-toxic to the body.

[0163] The bioresorbable region may be either hydrophobic or hydrophilic, so long as the copolymer composition as a whole is not rendered water-soluble. Thus, the bioresorbable

region is selected based on the preference that the polymer, as a whole, remains water-insoluble. Accordingly, the relative properties, *i.e.*, the kinds of functional groups contained by, and the relative proportions of the bioresorbable region, and the hydrophilic region are selected to ensure that useful bioresorbable compositions remain water-insoluble.

[0164] Exemplary resorbable polymers include, for example, synthetically produced resorbable block copolymers of poly(α-hydroxy-carboxylic acid)/poly(oxyalkylene, (*see*, Cohn *et al.*, U.S. Patent No. 4,826,945). These copolymers are not crosslinked and are water-soluble so that the body can excrete the degraded block copolymer compositions. *See*, Younes *et al.*, *J Biomed. Mater. Res.* **21**: 1301-1316 (1987); and Cohn *et al.*, *J Biomed. Mater. Res.* **22**: 993-1009 (1988).

[0165] Presently preferred bioresorbable polymers include one or more components selected from poly(esters), poly(hydroxy acids), poly(lactones), poly(amides), poly(ester-amides), poly (amino acids), poly(anhydrides), poly(orthoesters), poly(carbonates), poly(phosphazines), poly(phosphoesters), poly(thioesters), polysaccharides and mixtures thereof. More preferably still, the biosresorbable polymer includes a poly(hydroxy) acid component. Of the poly(hydroxy) acids, polylactic acid, polyglycolic acid, polycaproic acid, polybutyric acid, polyvaleric acid and copolymers and mixtures thereof are preferred.

[0166] In addition to forming fragments that are absorbed *in vivo* ("bioresorbed"), preferred polymeric coatings for use in the methods of the invention can also form an excretable and/or metabolizable fragment.

[0167] Higher order copolymers can also be used in the present invention. For example, Casey *et al.*, U.S. Patent No. 4,438,253, which issued on March 20, 1984, discloses tri-block copolymers produced from the transesterification of poly(glycolic acid) and an hydroxyl-ended poly(alkylene glycol). Such compositions are disclosed for use as resorbable monofilament sutures. The flexibility of such compositions is controlled by the incorporation of an aromatic orthocarbonate, such as tetra-p-tolyl orthocarbonate into the copolymer structure.

[0168] Other polymers based on lactic and/or glycolic acids can also be utilized. For example, Spinu, U.S. Patent No. 5,202,413, which issued on April 13, 1993, discloses biodegradable multi-block copolymers having sequentially ordered blocks of polylactide and/or polyglycolide produced by ring-opening polymerization of lactide and/or glycolide

onto either an oligomeric diol or a diamine residue followed by chain extension with a di-functional compound, such as, a diisocyanate, diacylchloride or dichlorosilane.

[0169] Bioresorbable regions of coatings useful in the present invention can be designed to be hydrolytically and/or enzymatically cleavable. For purposes of the present invention, "hydrolytically cleavable" refers to the susceptibility of the copolymer, especially the bioresorbable region, to hydrolysis in water or a water-containing environment. Similarly, "enzymatically cleavable" as used herein refers to the susceptibility of the copolymer, especially the bioresorbable region, to cleavage by endogenous or exogenous enzymes.

[0170] When placed within the body, the hydrophilic region can be processed into excretable and/or metabolizable fragments. Thus, the hydrophilic region can include, for example, polyethers, polyalkylene oxides, polyols, poly(vinyl pyrrolidine), poly(vinyl alcohol), poly(alkyl oxazolines), polysaccharides, carbohydrates, peptides, proteins and copolymers and mixtures thereof. Furthermore, the hydrophilic region can also be, for example, a poly(alkylene) oxide. Such poly(alkylene) oxides can include, for example, poly(ethylene) oxide, poly(propylene) oxide and mixtures and copolymers thereof.

[0171] Polymers that are components of hydrogels are also useful in the present invention. Hydrogels are polymeric materials that are capable of absorbing relatively large quantities of water. Examples of hydrogel forming compounds include, but are not limited to, polyacrylic acids, sodium carboxymethylcellulose, polyvinyl alcohol, polyvinyl pyrrolidine, gelatin, carrageenan and other polysaccharides, hydroxyethylenemethacrylic acid (HEMA), as well as derivatives thereof, and the like. Hydrogels can be produced that are stable, biodegradable and bioresorbable. Moreover, hydrogel compositions can include subunits that exhibit one or more of these properties.

[0172] Bio-compatible hydrogel compositions whose integrity can be controlled through crosslinking are known and are presently preferred for use in the methods of the invention. For example, Hubbell *et al.*, U.S. Patent Nos. 5,410,016, which issued on April 25, 1995 and 5,529,914, which issued on June 25, 1996, disclose water-soluble systems, which are crosslinked block copolymers having a water-soluble central block segment sandwiched between two hydrolytically labile extensions. Such copolymers are further end-capped with photopolymerizable acrylate functionalities. When crosslinked, these systems become hydrogels. The water soluble central block of such copolymers can include poly(ethylene glycol); whereas, the hydrolytically labile extensions can be a poly(α-hydroxy acid), such as

polyglycolic acid or polylactic acid. *See*, Sawhney *et al.*, *Macromolecules* **26**: 581-587 (1993).

[0173] In another preferred embodiment, the gel is a thermoreversible gel. Thermoreversible gels including components, such as pluronics, collagen, gelatin, hyalouronic acid, polysaccharides, polyurethane hydrogel, polyurethane-urea hydrogel and combinations thereof are presently preferred.

[0174] In yet another exemplary embodiment, the conjugate of the invention includes a component of a liposome. Liposomes can be prepared according to methods known to those skilled in the art, for example, as described in Eppstein *et al.*, U.S. Patent No. 4,522,811, which issued on June 11, 1985. For example, liposome formulations may be prepared by dissolving appropriate lipid(s) (such as stearoyl phosphatidyl ethanolamine, stearoyl phosphatidyl choline, arachadoyl phosphatidyl choline, and cholesterol) in an inorganic solvent that is then evaporated, leaving behind a thin film of dried lipid on the surface of the container. An aqueous solution of the active compound or its pharmaceutically acceptable salt is then introduced into the container. The container is then swirled by hand to free lipid material from the sides of the container and to disperse lipid aggregates, thereby forming the liposomal suspension.

[0175] The above-recited microparticles and methods of preparing the microparticles are offered by way of example and they are not intended to define the scope of microparticles of use in the present invention. It will be apparent to those of skill in the art that an array of microparticles, fabricated by different methods, are of use in the present invention.

Biomolecules

[0176] In another exemplary embodiment, the modified saccharyl fragment bears a biomolecule. In still further preferred embodiments, the biomolecule is a functional protein, enzyme, antigen, antibody, peptide, nucleic acid (*e.g.*, single nucleotides or nucleosides, oligonucleotides, polynucleotides and single- and higher-stranded nucleic acids), lectin, receptor or a combination thereof.

[0177] In a presently preferred embodiment, the modifying group is biotin. In an exemplary embodiment, the selectively biotinylated peptide is elaborated by the attachment of an avidin or streptavidin moiety bearing one or more modifying groups. Preferred biomolecules are essentially non-fluorescent, or emit such a minimal amount of fluorescence that they are inappropriate for use as a fluorescent marker in an assay. Moreover, it is

generally preferred to use biomolecules that are not sugars. An exception to this preference is the use of an otherwise naturally occurring sugar that is modified by covalent attachment of another entity (*e.g.*, PEG, biomolecule, therapeutic moiety, diagnostic moiety, *etc.*). In an exemplary embodiment, a sugar moiety, which is a biomolecule, is conjugated to a linker arm and the sugar-linker arm cassette is subsequently conjugated to a peptide via a method of the invention.

[0178] Biomolecules useful in practicing the present invention can be derived from any source. The biomolecules can be isolated from natural sources or they can be produced by synthetic methods. Peptides can be natural peptides or mutated peptides. Mutations can be effected by chemical mutagenesis, site-directed mutagenesis or other means of inducing mutations known to those of skill in the art. Peptides useful in practicing the instant invention include, for example, enzymes, antigens, antibodies and receptors. Antibodies can be either polyclonal or monoclonal.

[0179] Both naturally derived and synthetic peptides and nucleic acids are of use in conjunction with the present invention; these molecules can be attached to a sugar residue component or a crosslinking agent by any available reactive group. For example, peptides can be attached through a reactive amine, carboxyl, sulfhydryl, or hydroxyl group. The reactive group can reside at a peptide terminus or at a site internal to the peptide chain. Nucleic acids can be attached through a reactive group on a base (*e.g.*, exocyclic amine) or an available hydroxyl group on a sugar moiety (*e.g.*, 3'- or 5'-hydroxyl). The peptide and nucleic acid chains can be further derivatized at one or more sites to allow for the attachment of appropriate reactive groups onto the chain. *See,* Chrisey *et al. Nucleic Acids Res.* **24:** 3031-3039 (1996).

[0180] In a further preferred embodiment, the biomolecule is selected to direct the peptide modified by the methods of the invention to a specific tissue, thereby enhancing the delivery of the peptide to that tissue relative to the amount of underivatized peptide that is delivered to the tissue. In a still further preferred embodiment, the amount of derivatized peptide delivered to a specific tissue within a selected time period is enhanced by derivatization by at least about 20%, more preferably, at least about 40%, and more preferably still, at least about 100%. Presently, preferred biomolecules for targeting applications include antibodies, hormones and ligands for cell-surface receptors.

The Methods

[0181] In addition to the compositions discussed above, the present invention provides methods for preparing modified saccharyl fragments and glyco-conjugates incorporating these fragments. Exemplary methods include synthesizing a modified peptide or lipid using a modified saccharyl fragment, *e.g.*, modified-galactose, -fucose, and –sialic acid. When a modified sialic acid is used, either a sialyltransferase or a trans-sialidase (for α2,3-linked sialic acid only) can be used to transfer the modified fragment onto the acceptor moiety of the substrate.

[0182] The method of the invention includes transferring a modified saccharyl fragment from an activated modified saccharyl fragment onto an acceptor moiety of a substrate. Exemplary substrates include peptides and lipids of therapeutic relevance. Exemplary acceptor moieties include amino acid residues, aglycone residues and glycosyl moieties directly or indirectly bound to an amino acid or aglycone residue.

[0183] For clarity of illustration, the invention is illustrated with reference to a conjugate formed between a (glyco)peptide a modified saccharyl fragment that is transferred to an acceptor moiety on the (glyco)peptide from an activated modified saccharyl fragment that includes a water-soluble polymer. Those of skill will appreciate that the invention equally encompasses methods of forming conjugates of (glyco)lipids with saccharyl fragments modified with water-soluble polymers, and forming conjugates between (glyco)peptides and (glyco)lipids and saccharyl fragments bearing modifying groups other than water-soluble polymers.

[0184] In exemplary embodiments, the conjugate is formed between a water-soluble polymer, a therapeutic moiety, targeting moiety or a biomolecule, and a glycosylated peptide. The polymer, therapeutic moiety or biomolecule is conjugated to the peptide via a glycosyl linking group, which is interposed between, and covalently linked to, both the peptide (directly or through an intervening glycosyl linker) and the modifying group (*e.g.*, water-soluble polymer). The glycosyl linking group includes a modified saccharyl fragment. The method includes contacting the glycopeptide with an activated modified saccharyl fragment and an enzyme for which the activated modified saccharyl fragment is a substrate. The components of the reaction mixture are combined under conditions appropriate to enzymatically tranfer the modified saccharyl fragment from the activated modified saccharyl fragment to an acceptor moiety on the glycopeptide, thereby preparing the conjugate.

[0185] The acceptor peptide is typically synthesized *de novo*, or recombinantly expressed in a prokaryotic cell (*e.g.*, bacterial cell, such as *E. coli*) or in a eukaryotic cell such as a mammalian, yeast, insect, fungal or plant cell. The peptide can be either a full-length protein or a fragment. Moreover, the peptide can be a wild type or mutated peptide. In an exemplary embodiment, the peptide includes a mutation that adds one or more N- or O-linked glycosylation sites to the peptide sequence.

[0186] The method of the invention also provides for modification of incompletely glycosylated peptides that are produced recombinantly. Many recombinantly produced glycoproteins are incompletely glycosylated, exposing carbohydrate residues that may have undesirable properties, *e.g.*, immunogenicity, recognition by the RES. The incomplete glycosyl residue can be masked using a water-soluble polymer.

[0187] Exemplary peptides that can be modified using the methods of the invention are set forth in Table 1.

Table 1

Hormones and Growth Factors • G-CSF • GM-CSF • M-CSF • TPO • EPO • EPO variants • alpha-TNF • Leptin • FSH • HGH • FGH • GLP Enzymes and Inhibitors • t-PA • t-PA variants • Urokinase • Factors VII, VIIa, VIII, IX, X • Dnase • Glucocerebrosidase • Hirudin • α1 antitrypsin • Antithrombin III • α-galactosidase Cytokines and Chimeric Cytokines	Receptors and Chimeric Receptors • CD4 • Tumor Necrosis Factor (TNF) receptor • Alpha-CD20 • MAb-CD20 • MAb-alpha-CD3 • MAb-TNF receptor • MAb-CD4 • PSGL-1 • MAb-PSGL-1 • Complement • GlyCAM or its chimera • N-CAM or its chimera • Monoclonal Antibodies (Immunoglobulins) • MAb-anti-RSV • MAb-anti-IL-2 receptor • MAb-anti-CEA • MAb-anti-platelet IIb/IIIa receptor • MAb-anti-EGF • MAb-anti-Her-2 receptor Cells • Red blood cells • White blood cells (*e.g.*, T cells, B cells, dendritic cells, macrophages, NK cells, neutrophils, monocytes and the like

• Interleukin-1 (IL-1), 1B, 2, 3, 4 • Interferon-alpha (IFN-alpha) • IFN-alpha-2b • IFN-beta • IFN-gamma • IFN-omega • Chimeric diptheria toxin-IL-2	• Stem cells

[0188] Other exemplary peptides that are modified by the methods of the invention include members of the immunoglobulin family (*e.g.*, antibodies, MHC molecules, T cell receptors, and the like), intercellular receptors (*e.g.*, integrins, receptors for hormones or growth factors and the like) lectins, and cytokines (*e.g.*, interleukins). Additional examples include tissue-type plasminogen activator (t-PA), renin, clotting factors such as factors V-XII, bombesin, thrombin, hematopoietic growth factor, colony stimulating factors, viral antigens, complement proteins, α1-antitrypsin, erythropoietin, P-selectin glycopeptide ligand-1 (PSGL-1), granulocyte-macrophage colony stimulating factor, anti-thrombin III, interleukins, interferons, proteins A and C, fibrinogen, herceptin, leptin, glycosidases, HS-glycoprotein, serum proteins (e.g., α-acid glycoprotein, fetuin, α-fetal protein), β2-glycoprotein, among many others. This list of polypeptides is exemplary, not exclusive. The methods are also useful for modifying fusion and chimeric proteins, including, but not limited to, chimeric proteins that include a moiety derived from an immunoglobulin, such as IgG, or a fragment of an immunoglobin, *e.g.*, FAb (Fc domain). The exemplary peptides provided herein are intended to provide a selection of the peptides with which the present invention can be practiced; as such, they are non-limiting. Those of skill will appreciate that the invention can be practiced using substantially any peptide from any source. See, for example, U.S. Patent Publication 2004/0142856 and WO 03/031464.

[0189] Peptides modified by the methods of the invention can be synthetic or wild-type peptides or they can be mutated peptides, produced by methods known in the art, such as site-directed mutagenesis. Glycosylation of peptides is typically either N-linked or O-linked. An exemplary N-linkage is the attachment of the modified saccharyl fragment to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of a carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one sugar (*e.g.*, N-acetylgalactosamine, galactose, mannose, GlcNAc, glucose, fucose or xylose) to the hydroxy

side chain of a hydroxyamino acid, preferably serine or threonine, although unusual or non-natural amino acids, e.g., 5-hydroxyproline or 5-hydroxylysine may also be used.

[0190] Moreover, in addition to peptides, the methods of the present invention can be practiced with other biological structures (*e.g.*, glycolipids, lipids, sphingoids, ceramides, whole cells, and the like. In general, the only limitation on the substrate structure is that it includes a glycosylation site).

[0191] For substrates lacking a glycosylation site, or for which it is desired to add a further glycosylation site, reliable methods are known in the art. For example, addition of glycosylation sites to a peptide, or other structure, is conveniently accomplished by altering the amino acid sequence such that it contains the desired glycosylation site. The addition may be made by mutation or by full chemical synthesis of the peptide. The peptide amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the peptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) are preferably made using methods known in the art. Both O-linked and N-linked glycosylation sites can be engineered into a peptide.

[0192] In an exemplary embodiment, the glycosylation site is added by shuffling polynucleotides. Polynucleotides encoding a candidate peptide can be modulated with DNA shuffling protocols. DNA shuffling is a process of recursive recombination and mutation, performed by random fragmentation of a pool of related genes, followed by reassembly of the fragments by a polymerase chain reaction-like process. *See, e.g.*, Stemmer, *Proc. Natl. Acad. Sci. USA* **91**: 10747-10751 (1994); Stemmer, *Nature* **370**: 389-391 (1994); and U.S. Patent Nos. 5,605,793, 5,837,458, 5,830,721 and 5,811,238.

[0193] The present invention also provides means of adding (or removing) one or more selected glycosyl residues to a peptide, after which a modified saccharyl fragment is conjugated to at least one of the selected glycosyl residues of the peptide. The present embodiment is useful, for example, when it is desired to conjugate the modified saccharyl fragment to a selected glycosyl residue that is either not present on a peptide or is not present in a desired amount. Thus, prior to coupling a modified saccharyl fragment to a peptide, the selected glycosyl residue is conjugated to the peptide by enzymatic or chemical coupling. In another embodiment, the glycosylation pattern of a glycopeptide is altered prior to the

conjugation of the modified saccharyl fragment by the removal of a carbohydrate residue from the glycopeptide. *See*, for example WO 98/31826.

[0194] Addition or removal of any carbohydrate moiety present on the glycopeptide is accomplished either chemically or enzymatically. Chemical deglycosylation is preferably brought about by exposure of the polypeptide variant to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the peptide intact. Chemical deglycosylation is described by Hakimuddin *et al., Arch. Biochem. Biophys.* **259**: 52 (1987) and by Edge *et al., Anal. Biochem.* **118**: 131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptide variants can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al., Meth. Enzymol.* **138**: 350 (1987).

[0195] Chemical addition of glycosyl moieties is carried out by any art-recognized method. Enzymatic addition of sugar moieties is preferably achieved using a modification of the methods set forth herein, substituting native glycosyl units for the modified saccharyl fragments used in the invention. Other methods of adding sugar moieties are disclosed in U.S. Patent No. 5,876,980, 6,030,815, 5,728,554, and 5,922,577.

[0196] Exemplary attachment points for selected glycosyl residue include, but are not limited to: (a) consensus sites for N-linked glycosylation, and sites for O-linked glycosylation; (b) terminal glycosyl moieties that are acceptors for a glycosyltransferase; (c) arginine, asparagine and histidine; (d) free carboxyl groups; (e) free sulfhydryl groups such as those of cysteine; (f) free hydroxyl groups such as those of serine, threonine, or hydroxyproline; (g) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan; or (h) the amide group of glutamine. Exemplary methods of use in the present invention are described in WO 87/05330 published Sep. 11, 1987, and in Aplin and Wriston, CRC CRIT. REV. BIOCHEM., pp. 259-306 (1981).

[0197] In one embodiment, the invention provides a method for linking two or more peptides through a linking group. The linking group is of any useful structure and may be selected from straight- and branched-chain structures. Preferably, each terminus of the linker, which is attached to a peptide, includes a modified saccharyl fragment.

[0198] In an exemplary method of the invention, two peptides are linked together via a linker moiety that includes a polymeric (e.g., PEG linker). The focus on a PEG linker that

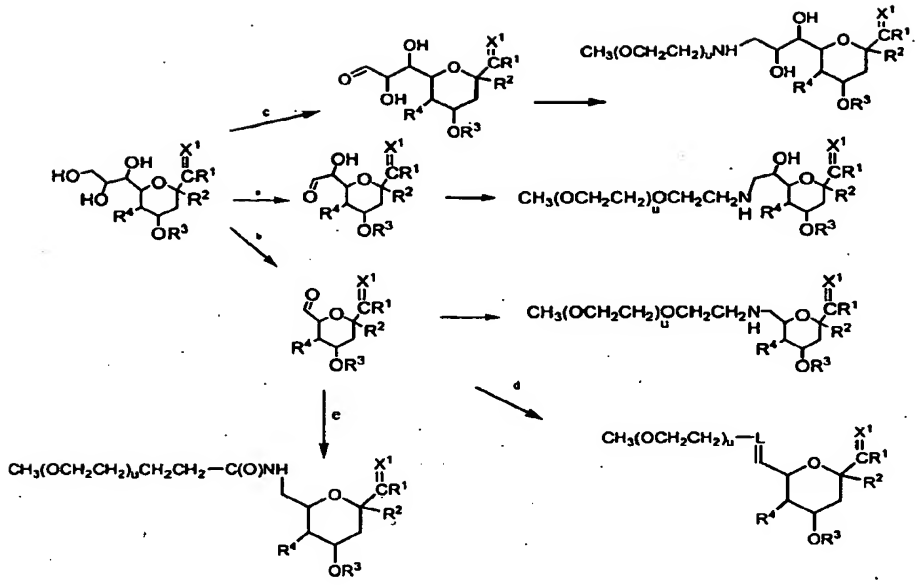
includes two glycosyl groups is for purposes of clarity and should not be interpreted as limiting the identity of linker arms of use in this embodiment of the invention. In an example of this embodiment, diamino-PEG is converted to a bifunctional linking group by reaction with two saccharyl fragments, e.g., sialic acid aldehyde. The bifunctional linking group is then enzymatically coupled to each peptide. As will be appreciated by those of skill in the art, the saccharyl fragments attached to the PEG moiety can be the same or different.

[0199] Exemplary peptides with which the present invention can be practiced, methods of adding or removing glycosylation sites, and adding or removing glycosyl structures or substructures are described in detail in WO03/031464 and related U.S. and PCT applications.

Preparation of Modified Saccharyl Fragments

[0200] In general, the saccharyl fragment and the modifying group are linked together through the use of reactive groups, which are typically transformed by the linking process into a new organic functional group or unreactive species. The reactive group on the saccharyl fragment in generally formed through a degradative process, e.g., oxidation. In the present invention, the modified saccharyl fragment is generally made by combining an amino analogue of the modifying group with an aldehyde or ketone moiety generated by oxidation of a saccharyl hydroxyl moiety.

[0201] Methods for converting saccharyl hydroxyl moieties into carbonyl-containing compounds are well known in the art. As exemplified by the selective oxidation of the side chain of sialic acid, conditions are generally available for preparing an oxidized saccharyl precursor in a controlled and reproducible fashion.



[0202] For example, in the scheme above, selective oxidation of the primary hydroxyl of the sialic acid side chain, followed by reductive amination with m-PEG-NH_2 provides the corresponding saccharyl PEG-amine fragment according to route **c**.

[0203] Further, mild periodate oxidation (e.g., 1 mM sodium metaperiodate, 0 °C), according to route **a**, produces a sialic acid fragment that is incompletely oxidized relative to the fragment resulting from the harsher oxidation conditions of route **b**. The aldehyde is coupled with a modifying group, e.g., amino-m-PEG, under reducing conditions, thereby forming an exemplary sialic acid fragment-m-PEG conjugate.

[0204] As shown in route **d**, the oxidized sialic acid can also be reacted with a Wittig, Grignard or lithium reagent to form a species in which the water-soluble polymer and the saccharyl fragment are conjugated through a linker group, L. The alkene moiety can be reduced using art-recognized conditions, forming a species in which L is linked to the remainder of the saccharyl fragment through a saturated C-C bond. Exemplary linkers include substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl moieties.

[0205] Route **e** exemplifies a scheme in which the aldhehyde is reductively aminated with ammonia and the resulting amine is acylated with an active m-PEG derivative, e.g., an active ester.

[0206] Those of skill in the art will readily appreciate that both routes **d** and **e** can be practiced with any of the side chain oxidized sialic acid fragments set forth in the scheme.

[0207] In addition to the species described above, R^1-R^4 can also represent or include protecting groups or protected groups. Those of skill in the art understand how to protect a particular functional group such that it does not interfere with a chosen set of reaction conditions. For examples of useful protecting groups, *see,* for example, Greene *et al.*, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS, John Wiley & Sons, New York, 1991.

[0208] Although exemplified above by reference to the use of an amine analogue of the modifying group, it is understood that the aldehyde or ketone group of the saccharide is readily modified by via formation of carbonyl derivatives such as, for example, imines, hydrazones, semicarbazones or oximes, or via such mechanisms as Grignard addition or alkyllithium addition. Accordingly, the present invention encompasses modified saccharyl fragments, linking groups and conjugates that include one or more of these derivatives, and is not limited to a particular saccharyl fragment or method of forming the fragment.

[0209] Exemplary moieties attached to the conjugates disclosed herein include, but are not limited to, PEG derivatives (*e.g.*, acyl-PEG, acyl-alkyl-PEG, alkyl-acyl-PEG carbamoyl-PEG, aryl-PEG), PPG derivatives (*e.g.*, acyl-PPG, acyl-alkyl-PPG, alkyl-acyl-PPG carbamoyl-PPG, aryl-PPG), therapeutic moieties, diagnostic moieties, mannose-6-phosphate, heparin, heparan, SLe_x, mannose, mannose-6-phosphate, Sialyl Lewis X, FGF, VFGF, proteins, chondroitin, keratan, dermatan, albumin, integrins, antennary oligosaccharides, peptides and the like. Methods of conjugating the various modifying groups to a saccharide moiety are readily accessible to those of skill in the art (POLY (ETHYLENE GLYCOL CHEMISTRY : BIOTECHNICAL AND BIOMEDICAL APPLICATIONS, J. Milton Harris, Ed., Plenum Pub. Corp., 1992; POLY (ETHYLENE GLYCOL) CHEMICAL AND BIOLOGICAL APPLICATIONS, J. Milton Harris, Ed., ACS Symposium Series No. 680, American Chemical Society, 1997; Hermanson, BIOCONJUGATE TECHNIQUES, Academic Press, San Diego, 1996; and Dunn *et al.*, Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991).

Cross-linking Groups

[0210] Preparation of the modified saccharyl fragment for use in the methods of the present invention includes attachment of a modifying group to a sugar residue and forming a stable

adduct, which is a substrate for a glycosyltransferase. Thus, it is often preferred to use a cross-linking agent to conjugate the modifying group and the sugar. Exemplary bifunctional compounds which can be used for attaching modifying groups to carbohydrate moieties include, but are not limited to, bifunctional poly(ethyleneglycols), polyamides, polyethers, polyesters and the like. General approaches for linking carbohydrates to other molecules are known in the literature. *See,* for example, Lee *et al.*, *Biochemistry* **28**: 1856 (1989); Bhatia *et al.*, *Anal. Biochem.* **178**: 408 (1989); Janda *et al.*, *J. Am. Chem. Soc.* **112**: 8886 (1990) and Bednarski *et al.*, WO 92/18135. In the discussion that follows, the reactive groups are treated as benign on the sugar moiety of the nascent modified saccharyl fragment. The focus of the discussion is for clarity of illustration. Those of skill in the art will appreciate that the discussion is relevant to reactive groups on the modifying group as well.

[0211] A variety of reagents are used to modify the components of the modified saccharyl fragment with intramolecular chemical crosslinks (for reviews of crosslinking reagents and crosslinking procedures see: Wold, F., *Meth. Enzymol.* **25**: 623-651, 1972; Weetall, H. H., and Cooney, D. A., In: ENZYMES AS DRUGS. (Holcenberg, and Roberts, eds.) pp. 395-442, Wiley, New York, 1981; Ji, T. H., *Meth. Enzymol.* **91**: 580-609, 1983; Mattson *et al.*, *Mol. Biol. Rep.* **17**: 167-183, 1993, all of which are incorporated herein by reference). Preferred crosslinking reagents are derived from various zero-length, homo-bifunctional, and hetero-bifunctional crosslinking reagents. Zero-length crosslinking reagents include direct conjugation of two intrinsic chemical groups with no introduction of extrinsic material. Agents that catalyze formation of a disulfide bond belong to this category. Another example is reagents that induce condensation of a carboxyl and a primary amino group to form an amide bond such as carbodiimides, ethylchloroformate, Woodward's reagent K (2-ethyl-5-phenylisoxazolium-3'-sulfonate), and carbonyldiimidazole. In addition to these chemical reagents, the enzyme transglutaminase (glutamyl-peptide γ-glutamyltransferase; EC 2.3.2.13) may be used as zero-length crosslinking reagent. This enzyme catalyzes acyl transfer reactions at carboxamide groups of protein-bound glutaminyl residues, usually with a primary amino group as substrate. Preferred homo- and hetero-bifunctional reagents contain two identical or two dissimilar sites, respectively, which may be reactive for amino, sulfhydryl, guanidino, indole, or nonspecific groups.

[0212] An exemplary cross-linking moiety includes a reactive functional group that reacts with the saccharyl ketone or aldehyde moiety (e.g., amine, hydrazine, etc.). The reactive functional group is tethered to a second reactive functional group that reacts with a moiety on

the modifying group, forming a linker covalently bonded to both the saccharyl fragment and the modifying group.

[0213] Exemplary cross-linking groups of use in the present invention are set forth in WO03/031464 and related U.S. and PCT applications.

Conjugation of Modified Saccharyl Fragments to Peptides

[0214] The modified saccharyl fragments are conjugated to a glycosylated or non-glycosylated peptide using an appropriate enzyme to mediate the conjugation. Preferably, the concentrations of the modified donor sugar(s), enzyme(s) and acceptor peptide(s) are selected such that glycosylation proceeds until the acceptor is consumed. The considerations discussed below, while set forth in the context of a sialyltransferase, are generally applicable to other glycosyltransferase reactions.

[0215] A number of methods of using glycosyltransferases to synthesize desired oligosaccharide structures are known and are generally applicable to the instant invention. Exemplary methods are described, for instance, WO 96/32491, Ito *et al.*, *Pure Appl. Chem.* 65: 753 (1993), and U.S. Pat. Nos. 5,352,670, 5,374,541, and 5,545,553.

[0216] The present invention is practiced using a single glycosyltransferase or a combination of glycosyltransferases. For example, one can use a combination of a sialyltransferase and a galactosyltransferase. In those embodiments using more than one enzyme, the enzymes and substrates are preferably combined in an initial reaction mixture, or the enzymes and reagents for a second enzymatic reaction are added to the reaction medium once the first enzymatic reaction is complete or nearly complete. By conducting two enzymatic reactions in sequence in a single vessel, overall yields are improved over procedures in which an intermediate species is isolated. Moreover, cleanup and disposal of extra solvents and by-products is reduced.

[0217] In a preferred embodiment, each of the first and second enzyme is a glycosyltransferase. In another preferred embodiment, one enzyme is an endoglycosidase. In an additional preferred embodiment, more than two enzymes are used to assemble the modified glycoprotein of the invention. The enzymes are used to alter a saccharide structure on the peptide at any point either before or after the addition of the modified saccharyl fragment to the peptide.

[0218] In another preferred embodiment, each of the enzymes utilized to produce a conjugate of the invention are present in a catalytic amount. The catalytic amount of a particular enzyme varies according to the concentration of that enzyme's substrate as well as to reaction conditions such as temperature, time and pH value. Means for determining the catalytic amount for a given enzyme under preselected substrate concentrations and reaction conditions are well known to those of skill in the art.

[0219] The temperature at which an above process is carried out can range from just above freezing to the temperature at which the most sensitive enzyme denatures. Preferred temperature ranges are about 0 °C to about 45 °C, and more preferably about 20 ° C to about 30 °C. In another exemplary embodiment, one or more components of the present method are conducted at an elevated temperature using a thermophilic enzyme.

[0220] The reaction mixture is maintained for a period of time sufficient for the acceptor to be glycosylated, thereby forming the desired conjugate. Some of the conjugate can often be detected after a few hours, with recoverable amounts usually being obtained within 24 hours or less. Those of skill in the art understand that the rate of reaction is dependent on a number of variable factors (*e.g*, enzyme concentration, donor concentration, acceptor concentration, temperature, solvent volume), which are optimized for a selected system.

[0221] The present invention also provides for the industrial-scale production of modified peptides.

[0222] In the discussion that follows, the invention is exemplified by the conjugation of modified sialic acid fragment to a glycosylated peptide. The exemplary modified sialic acid fragment is labeled with PEG. The focus of the following discussion on the use of PEG-modified sialic acid fragments and glycosylated peptides is for clarity of illustration and is not intended to imply that the invention is limited to the conjugation of these two partners. One of skill understands that the discussion is generally applicable to the additions of modified glycosyl fragments other than sialic acid fragments. Moreover, the discussion is equally applicable to the modification of a saccharyl fragment with agents other than PEG including other water-soluble polymers, therapeutic moieties, and biomolecules.

[0223] An enzymatic approach can be used for the selective introduction of PEGylated or PPGylated carbohydrates onto a peptide or glycopeptide. The method utilizes modified saccharyl fragments containing PEG, PPG, or a masked reactive functional group, and is combined with the appropriate glycosyltransferase or glycosynthase. By selecting the

glycosyltransferase that will make the desired carbohydrate linkage and utilizing the modified saccharyl fragment as the donor substrate, the PEG or PPG can be introduced directly onto the peptide backbone, onto existing sugar residues of a glycopeptide or onto sugar residues that have been added to a peptide.

[0224] An acceptor for the sialyltransferase is present on the peptide to be modified by the methods of the present invention either as a naturally occurring structure or one placed there recombinantly, enzymatically or chemically. Suitable acceptors, include, for example, galactosyl acceptors such as GalNAc, Galβ1,4GlcNAc, Galβ1,4GalNAc, Galβ1,3GalNAc, lacto-N-tetraose, Galβ1,3GlcNAc, Galβ1,3Ara, Galβ1,6GlcNAc, Galβ1,4Glc (lactose), and other acceptors known to those of skill in the art (*see, e.g.*, Paulson *et al., J. Biol. Chem.* **253**: 5617-5624 (1978)).

[0225] In one embodiment, an acceptor for the sialyltransferase is present on the glycopeptide to be modified upon *in vivo* synthesis of the glycopeptide. Such glycopeptides can be sialylated using the claimed methods without prior modification of the glycosylation pattern of the glycopeptide. Alternatively, the methods of the invention can be used to sialylate a peptide that does not include a suitable acceptor; one first modifies the peptide to include an acceptor by methods known to those of skill in the art. In an exemplary embodiment, a GalNAc residue is added by the action of a GalNAc transferase.

[0226] In an exemplary embodiment, an acceptor for a modified sialic acid fragment is assembled by attaching a galactose residue to an appropriate acceptor linked to the peptide, e.g., a GlcNAc. The method includes incubating the peptide to be modified with a reaction mixture that contains a suitable amount of a galactosyltransferase (*e.g.*, galβ1,3 or galβ1,4), and a suitable galactosyl donor (*e.g.*, UDP-galactose). The reaction is allowed to proceed substantially to completion or, alternatively, the reaction is terminated when a preselected amount of the galactose residue is added. Other methods of assembling a selected saccharide acceptor will be apparent to those of skill in the art.

[0227] In yet another embodiment, glycopeptide-linked oligosaccharides are first "trimmed," either in whole or in part, to expose either an acceptor for the sialyltransferase or a moiety to which one or more appropriate residues can be added to obtain a suitable acceptor. Enzymes such as glycosyltransferases and endoglycosidases (*see*, for example U.S. Patent No. 5,716,812) are useful for the attaching and trimming reactions.

[0228] In the discussion that follows, the method of the invention is exemplified by the use of modified saccharyl fragments having a water-soluble polymer attached thereto. The focus of the discussion is for clarity of illustration. Those of skill will appreciate that the discussion is equally relevant to those embodiments in which the modified saccharyl fragment bears a therapeutic moiety, biomolecule or the like.

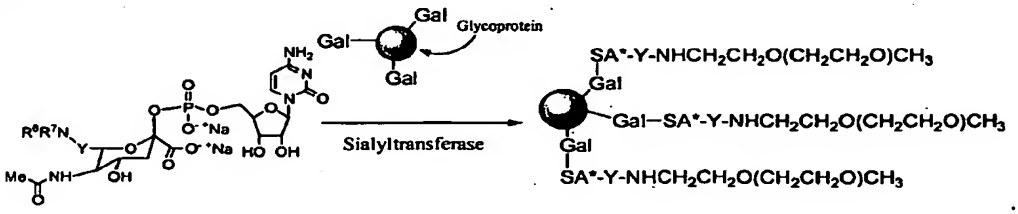
[0229] In another exemplary embodiment, a water-soluble polymer is added to one or both of the terminal mannose residues of the biantennary structure via a modified saccharyl fragment having a galactose residue, which is conjugated to a GlcNAc residue added onto the terminal mannose residues. Alternatively, an unmodified Gal can be added to one or both terminal GlcNAc residues.

[0230] In yet a further example, a water-soluble polymer is added onto a Gal residue using a modified sialic acid fragment.

[0231] The Examples set forth above provide an illustration of the power of the methods set forth herein. Using the methods of the invention, it is possible to "trim back" and build up a carbohydrate residue of substantially any desired structure. The modified saccharyl fragment can be added to the termini of the carbohydrate moiety as set forth above, or it can be intermediate between the peptide core and the terminus of the carbohydrate.

[0232] In an exemplary embodiment, an existing sialic acid is removed from a glycopeptide using a sialidase, thereby unmasking all or most of the underlying galactosyl residues. Alternatively, a peptide or glycopeptide is labeled with galactose residues, or an oligosaccharide residue that terminates in a galactose unit. Following the exposure of, or addition of, the galactose residues, an appropriate sialyltransferase is used to add a modified sialic acid. The approach is summarized in Scheme 2.

Scheme 2



In which SA* is saccharyl fragment and Y is as described above (Formula I).

[0233] In an alternative embodiment, the modified saccharyl fragment is added directly to the peptide backbone using a glycosyltransferase known to transfer sugar residues to the peptide backbone. Use of this approach allows the direct addition of modified saccharyl fragments onto peptides that lack any carbohydrates or, alternatively, onto existing glycopeptides. In both cases, the addition of the modified saccharyl fragment occurs at specific positions on the peptide backbone as defined by the substrate specificity of the glycosyltransferase and not in a random manner as occurs during modification of a protein's peptide backbone using chemical methods. An array of agents can be introduced into proteins or glycopeptides that lack the glycosyltransferase substrate peptide sequence by engineering the appropriate amino acid sequence into the polypeptide chain.

[0234] In each of the exemplary embodiments set forth above, one or more additional chemical or enzymatic modification steps can be utilized following the conjugation of the modified saccharyl fragment to the peptide. In an exemplary embodiment, an enzyme (*e.g.*, fucosyltransferase) is used to append a glycosyl unit (*e.g.*, fucose) onto the terminal modified saccharyl fragment attached to the peptide. In another example, an enzymatic reaction is utilized to "cap" sites to which the modified saccharyl fragment failed to conjugate. Alternatively, a chemical reaction is utilized to alter the structure of the conjugated modified saccharyl fragment. For example, the conjugated modified saccharyl fragment is reacted with agents that stabilize or destabilize its linkage with the peptide component to which the modified saccharyl fragment is attached. In another example, a component of the modified saccharyl fragment is deprotected following its conjugation to the peptide. One of skill will appreciate that there is an array of enzymatic and chemical procedures that are useful in the methods of the invention at a stage after the modified saccharyl fragment is conjugated to the peptide. Further elaboration of the modified saccharyl fragment-peptide conjugate is within the scope of the invention.

Enzymes

[0235] General methods of remodeling peptides and lipids using enzymes that transfer a sugar donor to an acceptor are discussed in detail in DeFrees, WO 03/031464 A2, published April 17, 2003. A brief summary of selected enzymes of use in the present method is set forth below.

Glycosyltransferases

[0236] Glycosyltransferases catalyze the addition of activated sugars (donor NDP-sugars), in a step-wise fashion, to a protein, glycopeptide, lipid or glycolipid or to the non-reducing end of a growing oligosaccharide. N-linked glycopeptides are synthesized via a transferase and a lipid-linked oligosaccharide donor Dol-PP-$NAG_2Glc_3Man_9$ in an en block transfer followed by trimming of the core. In this case the nature of the "core" saccharide is somewhat different from subsequent attachments. A very large number of glycosyltransferases are known in the art.

[0237] The glycosyltransferase to be used in the present invention may be any as long as it can utilize the modified saccharyl fragment as a sugar donor. Examples of such enzymes include Leloir pathway glycosyltransferase, such as galactosyltransferase, N-acetylglucosaminyltransferase, N-acetylgalactosaminyltransferase, fucosyltransferase, sialyltransferase, mannosyltransferase, xylosyltransferase, glucurononyltransferase and the like.

[0238] For enzymatic saccharide syntheses that involve glycosyltransferase reactions, glycosyltransferase can be cloned, or isolated from any source. Many cloned glycosyltransferases are known, as are their polynucleotide sequences. *See, e.g.*, "The WWW Guide To Cloned Glycosyltransferases," Taniguchi et al., 2002, Handbook of Glycosyltransferases and Related Genes, Springer, Tokyo. Glycosyltransferase amino acid sequences and nucleotide sequences encoding glycosyltransferases from which the amino acid sequences can be deduced are also found in various publicly available databases, including GenBank, Swiss-Prot, EMBL, and others.

[0239] Glycosyltransferases that can be employed in the methods of the invention include, but are not limited to, galactosyltransferases, fucosyltransferases, glucosyltransferases, N-acetylgalactosaminyltransferases, N-acetylglucosaminyltransferases, glucuronyltransferases, sialyltransferases, mannosyltransferases, glucuronic acid transferases, galacturonic acid transferases, and oligosaccharyltransferases. Suitable glycosyltransferases include those obtained from eukaryotes, as well as from prokaryotes. The enzymes may be wild-type or mutant enzymes. Methods of preparing mutant glycosyltransferases and characterizing these species are known in the art.

Fucosyltransferases

[0240] In some embodiments, a glycosyltransferase used in the method of the invention is a fucosyltransferase. Fucosyltransferases are known to those of skill in the art. Exemplary

fucosyltransferases include enzymes, which transfer L-fucose from GDP-fucose to a hydroxy position of an acceptor sugar. Fucosyltransferases that transfer non-nucleotide sugars to an acceptor are also of use in the present invention.

[0241] In some embodiments, the acceptor sugar is, for example, the GlcNAc in a Galβ(1→3,4)GlcNAcβ- group in an oligosaccharide glycoside. Suitable fucosyltransferases for this reaction include the Galβ(1→3,4)GlcNAcβ1-α(1→3,4)fucosyltransferase (FTIII E.C. No. 2.4.1.65), which was first characterized from human milk (*see*, Palcic, *et al.*, *Carbohydrate Res.* **190**: 1-11 (1989); Prieels, *et al., J. Biol. Chem.* **256**: 10456-10463 (1981); and Nunez, *et al., Can. J. Chem.* **59**: 2086-2095 (1981)) and the Galβ(1→4)GlcNAcβ-αfucosyltransferases (FTIV, FTV, FTVI) which are found in human serum. FTVII (E.C. No. 2.4.1.65), a sialyl α(2→3)Galβ((1→3)GlcNAcβ fucosyltransferase, has also been characterized. A recombinant form of the Galβ(1→3,4) GlcNAcβ-α(1→3,4)fucosyltransferase has also been characterized (*see*, Dumas, *et al., Bioorg. Med. Letters* **1**: 425-428 (1991) and Kukowska-Latallo, *et al., Genes and Development* **4**: 1288-1303 (1990)). Other exemplary fucosyltransferases include, for example, α1,2 fucosyltransferase (E.C. No. 2.4.1.69). Enzymatic fucosylation can be carried out by the methods described in Mollicone, *et al., Eur. J. Biochem.* **191**: 169-176 (1990) or U.S. Patent No. 5,374,655. Cells that are used to produce a fucosyltransferase will also include an enzymatic system for synthesizing GDP-fucose.

Galactosyltransferases

[0242] In another group of embodiments, the glycosyltransferase is a galactosyltransferase. Exemplary galactosyltransferases include α(1,3) galactosyltransferases (E.C. No. 2.4.1.151, *see, e.g.*, Dabkowski *et al., Transplant Proc.* **25**:2921 (1993) and Yamamoto *et al. Nature* **345**: 229-233 (1990), bovine (GenBank j04989, Joziasse *et al., J. Biol. Chem.* **264**: 14290-14297 (1989)), murine (GenBank m26925; Larsen *et al., Proc. Nat'l. Acad. Sci. USA* **86**: 8227-8231 (1989)), porcine (GenBank L36152; Strahan *et al., Immunogenetics* **41**: 101-105 (1995)). Another suitable α1,3 galactosyltransferase is that which is involved in synthesis of the blood group B antigen (EC 2.4.1.37, Yamamoto *et al., J. Biol. Chem.* **265**: 1146-1151 (1990) (human)). Yet a further exemplary galactosyltransferase is core Gal-T1.

[0243] Also suitable for use in the methods of the invention are β(1,4) galactosyltransferases, which include, for example, EC 2.4.1.90 (LacNAc synthetase) and EC 2.4.1.22 (lactose synthetase) (bovine (D'Agostaro *et al., Eur. J. Biochem.* **183**: 211-217

(1989)), human (Masri *et al., Biochem. Biophys. Res. Commun.* **157**: 657-663 (1988)), murine (Nakazawa *et al.,* J. Biochem. **104**: 165-168 (1988)), as well as E.C. 2.4.1.38 and the ceramide galactosyltransferase (EC 2.4.1.45, Stahl *et al., J. Neurosci. Res.* **38**: 234-242 (1994)). Other suitable galactosyltransferases include, for example, α1,2 galactosyltransferases (from *e.g., Schizosaccharomyces pombe*, Chapell *et al., Mol. Biol. Cell* **5**: 519-528 (1994)).

Sialyltransferases

[0244] Sialyltransferases are another type of glycosyltransferase that is useful in the recombinant cells and reaction mixtures of the invention. Cells that produce recombinant sialyltransferases will also produce CMP-sialic acid, which is a sialic acid donor for sialyltransferases. Examples of sialyltransferases that are suitable for use in the present invention include ST3Gal III (*e.g.*, a rat or human ST3Gal III), ST3Gal IV, ST3Gal I, ST6Gal I, ST3Gal V, ST6Gal II, ST6GalNAc I, ST6GalNAc II, and ST6GalNAc III (the sialyltransferase nomenclature used herein is as described in Tsuji *et al., Glycobiology* 6: v-xiv (1996)). An exemplary α(2,3)sialyltransferase referred to as α(2,3)sialyltransferase (EC 2.4.99.6) transfers sialic acid to the non-reducing terminal Gal of a Galβ1→3Glc disaccharide or glycoside. *See,* Van den Eijnden *et al., J. Biol. Chem.* **256:** 3159 (1981), Weinstein *et al., J. Biol. Chem.* **257:** 13845 (1982) and Wen *et al., J. Biol. Chem.* **267:** 21011 (1992). Another exemplary α2,3-sialyltransferase (EC 2.4.99.4) transfers sialic acid to the non-reducing terminal Gal of the disaccharide or glycoside. *see,* Rearick *et al., J. Biol. Chem.* **254:** 4444 (1979) and Gillespie *et al., J. Biol. Chem.* **267:** 21004 (1992). Further exemplary enzymes include Gal-β-1,4-GlcNAc α-2,6 sialyltransferase (*See,* Kurosawa *et al. Eur. J. Biochem.* **219:** 375-381 (1994)).

[0245] Preferably, for glycosylation of carbohydrates of glycopeptides the sialyltransferase will be able to transfer sialic acid to the sequence Galβ1,4GlcNAc-, the most common penultimate sequence underlying the terminal sialic acid on fully sialylated carbohydrate structures (*see,* Table 2, **FIG. 1**).

GalNAc transferases

[0246] N-acetylgalactosaminyltransferases are of use in practicing the present invention, particularly for binding a GalNAc moiety to an amino acid of the O-linked glycosylation site of the peptide. Suitable N-acetylgalactosaminyltransferases include, but are not limited to, α(1,3) N-acetylgalactosaminyltransferase, β(1,4) N-acetylgalactosaminyltransferases (Nagata *et al., J. Biol. Chem.* **267**: 12082-12089 (1992) and Smith *et al., J. Biol Chem.* **269**: 15162

(1994)) and polypeptide N-acetylgalactosaminyltransferase (Homa *et al.*, *J. Biol. Chem.* **268**: 12609 (1993)). See also the work of W. Wakarchuk generally and U.S. Patent No. 6,723,545; and published U.S. Patent Application No. 2003/0180928; 2003/0157658; 2003/0157657; and 2003/0157656.

[0247] Production of proteins such as the enzyme GalNAc T_{I-XX} from cloned genes by genetic engineering is well known. See, eg., U.S. Pat. No. 4,761,371. One method involves collection of sufficient samples, then the amino acid sequence of the enzyme is determined by N-terminal sequencing. This information is then used to isolate a cDNA clone encoding a full-length (membrane bound) transferase which upon expression in the insect cell line Sf9 resulted in the synthesis of a fully active enzyme. The acceptor specificity of the enzyme is then determined using a semiquantitative analysis of the amino acids surrounding known glycosylation sites in 16 different proteins followed by in vitro glycosylation studies of synthetic peptides. This work has demonstrated that certain amino acid residues are overrepresented in glycosylated peptide segments and that residues in specific positions surrounding glycosylated serine and threonine residues may have a more marked influence on acceptor efficiency than other amino acid moieties.

Cell-Bound Glycosyltransferases

[0248] In another embodiment, the enzymes utilized in the method of the invention are cell-bound glycosyltransferases. Although many soluble glycosyltransferases are known (see, for example, U.S. Pat. No. 5,032,519), glycosyltransferases are generally in membrane-bound form when associated with cells. Many of the membrane-bound enzymes studied thus far are considered to be intrinsic proteins; that is, they are not released from the membranes by sonication and require detergents for solubilization. Surface glycosyltransferases have been identified on the surfaces of vertebrate and invertebrate cells, and it has also been recognized that these surface transferases maintain catalytic activity under physiological conditions. However, the more recognized function of cell surface glycosyltransferases is for intercellular recognition (Roth, MOLECULAR APPROACHES to SUPRACELLULAR PHENOMENA, 1990).

[0249] Methods have been developed to alter the glycosyltransferases expressed by cells. For example, Larsen *et al.*, *Proc. Natl. Acad. Sci. USA* **86**: 8227-8231 (1989), report a genetic approach to isolate cloned cDNA sequences that determine expression of cell surface oligosaccharide structures and their cognate glycosyltransferases. A cDNA library generated from mRNA isolated from a murine cell line known to express UDP-galactose:.β.-D-

galactosyl-1,4-N-acetyl-D-glucosaminide α-1,3-galactosyltransferase was transfected into COS-1 cells. The transfected cells were then cultured and assayed for α 1-3 galactosyltransferase activity.

[0250] Francisco *et al., Proc. Natl. Acad. Sci. USA* **89**: 2713-2717 (1992), disclose a method of anchoring β-lactamase to the external surface of *Escherichia coli*. A tripartite fusion consisting of (i) a signal sequence of an outer membrane protein, (ii) a membrane-spanning section of an outer membrane protein, and (iii) a complete mature β-lactamase sequence is produced resulting in an active surface bound β-lactamase molecule. However, the Francisco method is limited only to procaryotic cell systems and as recognized by the authors, requires the complete tripartite fusion for proper functioning.

Sulfotransferases

[0251] The invention also provides methods for producing peptides that include sulfated molecules, including, for example sulfated polysaccharides such as heparin, heparan sulfate, carragenen, and related compounds. Suitable sulfotransferases include, for example, chondroitin-6-sulphotransferase (chicken cDNA described by Fukuta *et al., J. Biol. Chem.* **270**: 18575-18580 (1995); GenBank Accession No. D49915), glycosaminoglycan N-acetylglucosamine N-deacetylase/N-sulphotransferase 1 (Dixon *et al., Genomics* **26**: 239-241 (1995); UL18918), and glycosaminoglycan N-acetylglucosamine N-deacetylase/N-sulphotransferase 2 (murine cDNA described in Orellana *et al., J. Biol. Chem.* **269**: 2270-2276 (1994) and Eriksson *et al., J. Biol. Chem.* **269**: 10438-10443 (1994); human cDNA described in GenBank Accession No. U2304).

Glycosidases

[0252] This invention also encompasses the use of wild-type and mutant glycosidases. Mutant β-galactosidase enzymes have been demonstrated to catalyze the formation of disaccharides through the coupling of an α-glycosyl fluoride to a galactosyl acceptor molecule. (Withers, U.S. Pat. No. 6,284,494; issued Sept. 4, 2001). Other glycosidases of use in this invention include, for example, β-glucosidases, β-galactosidases, β-mannosidases, β-acetyl glucosaminidases, β-N-acetyl galactosaminidases, β-xylosidases, β-fucosidases, cellulases, xylanases, galactanases, mannanases, hemicellulases, amylases, glucoamylases, α-glucosidases, α-galactosidases, α-mannosidases, α-N-acetyl glucosaminidases, α-N-acetyl galactose-aminidases, α-xylosidases, α-fucosidases, and neuraminidases/sialidases.

Immobilized Enzymes

[0253] The present invention also provides for the use of enzymes that are immobilized on a solid and/or soluble support. In an exemplary embodiment, there is provided a glycosyltransferase that is conjugated to a PEG via an intact glycosyl linker according to the methods of the invention. The PEG-linker-enzyme conjugate is optionally attached to solid support. The use of solid supported enzymes in the methods of the invention simplifies the work up of the reaction mixture and purification of the reaction product, and also enables the facile recovery of the enzyme. The glycosyltransferase conjugate is utilized in the methods of the invention. Other combinations of enzymes and supports will be apparent to those of skill in the art.

Purification of Peptide Conjugates

[0254] The products produced by the above processes can be used without purification. However, it is usually preferred to recover the product. Standard, well-known techniques for recovery of modified peptides such as thin or thick layer chromatography, column chromatography, ion exchange chromatography, or membrane filtration can be used. It is preferred to use membrane filtration, more preferably utilizing a reverse osmotic membrane, or one or more column chromatographic techniques for the recovery as is discussed hereinafter and in the literature cited herein. For instance, membrane filtration wherein the membranes have molecular weight cutoff of about 3000 to about 10,000 can be used to remove proteins such as glycosyl transferases. Nanofiltration or reverse osmosis can then be used to remove salts and/or purify the conjugates (*see, e.g.,* WO 98/15581). Nanofilter membranes are a class of reverse osmosis membranes that pass monovalent salts but retain polyvalent salts and uncharged solutes larger than about 100 to about 2,000 Daltons, depending upon the membrane used. Thus, in a typical application, conjugates prepared by the methods of the present invention will be retained in the membrane and contaminating salts will pass through.

[0255] If the modified glycoprotein is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration; optionally, the protein may be concentrated with a commercially available protein concentration filter, followed by separating the polypeptide variant from other impurities by one or more steps selected from immunoaffinity chromatography, ion-exchange column fractionation (*e.g.*, on diethylaminoethyl (DEAE) or

matrices containing carboxymethyl or sulfopropyl groups), chromatography on Blue-Sepharose, CM Blue-Sepharose, MONO-Q, MONO-S, lentil lectin-Sepharose, WGA-Sepharose, Con A-Sepharose, Ether Toyopearl, Butyl Toyopearl, Phenyl Toyopearl, or protein A Sepharose, SDS-PAGE chromatography, silica chromatography, chromatofocusing, reverse phase HPLC (*e.g.*, silica gel with appended aliphatic groups), gel filtration using, *e.g.*, Sephadex molecular sieve or size-exclusion chromatography, chromatography on columns that selectively bind the polypeptide, and ethanol or ammonium sulfate precipitation.

[0256] Modified glycopeptides produced in culture are usually isolated by initial extraction from cells, enzymes, etc., followed by one or more concentration, salting-out, aqueous ion-exchange, or size-exclusion chromatography steps. Additionally, the modified glycoprotein may be purified by affinity chromatography. Finally, HPLC may be employed for final purification steps.

[0257] A protease inhibitor, *e.g.*, methylsulfonylfluoride (PMSF) may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

[0258] In another method, supernatants from systems that produce the modified glycopeptide of the invention are first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate may be applied to a suitable purification matrix. For example, a suitable affinity matrix may comprise a ligand for the peptide, a lectin or antibody molecule bound to a suitable support. Alternatively, an anion-exchange resin may be employed, for example, a matrix or substrate having pendant DEAE groups. Suitable matrices include acrylamide, agarose, dextran, cellulose, or other types commonly employed in protein purification. Alternatively, a cation-exchange step may be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are particularly preferred.

[0259] Finally, one or more RP-HPLC steps employing hydrophobic RP-HPLC media, *e.g.*, silica gel having pendant methyl or other aliphatic groups, may be employed to further purify a polypeptide variant composition. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a homogeneous modified glycoprotein.

[0260] The modified glycopeptide of the invention resulting from a large-scale fermentation may be purified by methods analogous to those disclosed by Urdal *et al., J. Chromatog.* **296**: 171 (1984). This reference describes two sequential, RP-HPLC steps for purification of recombinant human IL-2 on a preparative HPLC column. Alternatively, techniques such as affinity chromatography may be utilized to purify the modified glycoprotein.

Pharmaceutical Compositions

[0261] In another aspect, the invention provides a pharmaceutical composition. The pharmaceutical composition includes a pharmaceutically acceptable carrier and a covalent conjugate of the invention between water-soluble or –insoluble polymer, therapeutic moiety or biomolecule and a glycosylated or non-glycosylated peptide. The polymer, therapeutic moiety or biomolecule is conjugated to the peptide via an intact glycosyl linking group interposed between and covalently linked to both the peptide and the polymer, therapeutic moiety or biomolecule.

[0262] Pharmaceutical compositions of the invention are suitable for use in a variety of drug delivery systems. Suitable formulations for use in the present invention are found in *Remington's Pharmaceutical Sciences*, Mace Publishing Company, Philadelphia, PA, 17th ed. (1985). For a brief review of methods for drug delivery, *see*, Langer, *Science* **249**:1527-1533 (1990).

[0263] The pharmaceutical compositions may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, intravenous, intracranial, intraperitoneal, subcutaneous or intramuscular administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable microspheres (*e.g.*, polylactate polyglycolate) may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268 and 5,075,109.

[0264] Commonly, the pharmaceutical compositions are administered parenterally, *e.g.*, intravenously. Thus, the invention provides compositions for parenteral administration which comprise the compound dissolved or suspended in an acceptable carrier, preferably an

aqueous carrier, *e.g.*, water, buffered water, saline, PBS and the like. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents, detergents and the like.

[0265] These compositions may be sterilized by conventional sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous carrier prior to administration. The pH of the preparations typically will be between 3 and 11, more preferably from 5 to 9 and most preferably from 7 and 8.

[0266] In some embodiments the glycopeptides of the invention can be incorporated into liposomes formed from standard vesicle-forming lipids. A variety of methods are available for preparing liposomes, as described in, *e.g.*, Szoka *et al.*, *Ann. Rev. Biophys. Bioeng.* **9**: 467 (1980), U.S. Pat. Nos. 4,235,871, 4,501,728 and 4,837,028. The targeting of liposomes using a variety of targeting agents (*e.g.*, the sialyl galactosides of the invention) is well known in the art (*see, e.g.*, U.S. Patent Nos. 4,957,773 and 4,603,044).

[0267] Standard methods for coupling targeting agents to liposomes can be used. These methods generally involve incorporation into liposomes of lipid components, such as phosphatidylethanolamine, which can be activated for attachment of targeting agents, or derivatized lipophilic compounds, such as lipid-derivatized glycopeptides of the invention.

[0268] Targeting mechanisms generally require that the targeting agents be positioned on the surface of the liposome in such a manner that the target moieties are available for interaction with the target, for example, a cell surface receptor. The carbohydrates of the invention may be attached to a lipid molecule before the liposome is formed using methods known to those of skill in the art (*e.g.*, alkylation or acylation of a hydroxyl group present on the carbohydrate with a long chain alkyl halide or with a fatty acid, respectively). Alternatively, the liposome may be fashioned in such a way that a connector portion is first incorporated into the membrane at the time of forming the membrane. The connector portion must have a lipophilic portion, which is firmly embedded and anchored in the membrane. It must also have a reactive portion, which is chemically available on the aqueous surface of the liposome. The reactive portion is selected so that it will be chemically suitable to form a stable chemical bond with the targeting agent or carbohydrate, which is added later. In some cases it is possible to attach the target agent to the connector molecule directly, but in most

instances it is more suitable to use a third molecule to act as a chemical bridge, thus linking the connector molecule which is in the membrane with the target agent or carbohydrate which is extended, three dimensionally, off of the vesicle surface.

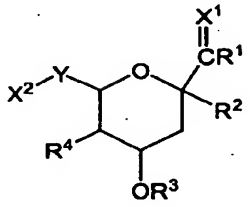
[0269] The compounds prepared by the methods of the invention may also find use as diagnostic reagents. For example, labeled compounds can be used to locate areas of inflammation or tumor metastasis in a patient suspected of having an inflammation. For this use, the compounds can be labeled with ^{125}I, ^{14}C, or tritium.

[0270] Moreover, the invention provides methods of preventing, curing or ameliorating a disease state by administering a conjugate of the invention to a subject at risk of developing the disease or to a subject that has the disease. The conjugate is administered in a therapeutically effective amount. Because many of the conjugates, particularly those that include a polymeric modifying group, are anticipated to display enhanced in vivo residence times, a therapeutically effective dosage is readily determinable from a dosage of the non-conjugated therapeutic agent typically administered.

[0271] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

WHAT IS CLAIMED IS:

1. A peptide conjugate, comprising the moiety:



wherein

X^1 is a member selected from O and NR^8

wherein

R^8 is a member selected from H, OH, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

Y is a member selected from CH_2, $CH(OH)CH_2$, $CH(OH)CH(OH)CH_2$, C, $CH(OH)C$ or $CH(OH)CH(OH)C$, C(OH), $CH(OH)CH(OH)$, and $CH(OH)CH(OH)C(OH)$;

X^2 is a member selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, NR^6R^7 and $R^6R^7N\text{-}N=$

wherein

R^6 and R^7 are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^1 and R^5 are members independently selected from OR^9, NR^9R^{10}, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl

wherein

R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and $C(O)R^{11}$

in which

R^{11} is selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl;

R^2 is a member selected from a nucleotide, an amino acid residue of a peptide, a carbohydrate moiety attached to an amino acid residue of a peptide, and a

carbohydrate moiety attached to an amino acid residue of a peptide through a linker comprising at least a second carbohydrate moiety;

R^3 is a member selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl; and

R^4 is a member selected from H, OH, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and $C(O)R^{12}$

in which

R^{12} is a member selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl and $NR^{13}R^{14}$

wherein

R^{13} and R^{14} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl.

2. The conjugate according to claim 1, wherein at least one of R^6 and R^7 comprises a modifying group.

3. The conjugate according to claim 2, wherein said modifying group is a member selected from straight- and branched-poly(ethylene glycol).

4. The conjugate according to claim 3, wherein at least one R^6 and R^7 has the formula:

$$-L-(m\text{-}PEG)_s$$

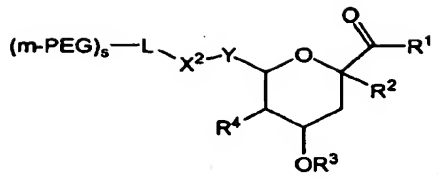
wherein

L is a bond or a linker moiety which is a member selected from an amino acid residue and a peptidyl residue; and

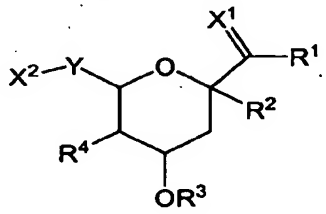
s is an integer from 1 to 3.

5. The conjugate according to claim 3, wherein said moiety is attached to a member selected from a carbohydrate moiety attached to an amino acid residue of said peptide, a carbohydrate moiety attached to an amino acid residue of said peptide through a linker comprising at least a second carbohydrate moiety.

6. The conjugate according to claim 1, wherein said moiety has the formula:



7. A compound having the formula:



wherein

X^1 is a member selected from O and NR^8

wherein

R^8 is a member selected from H, OH, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

Y is a member selected from CH_2, $CH(OH)CH_2$, $CH(OH)CH(OH)CH_2$, C, CH(OH)C or CH(OH)CH(OH)C, C(OH), CH(OH)CH(OH), and CH(OH)CH(OH)C(OH);

X^2 is a member selected from substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl, NR^6R^7 and R^6R^7N-N=

wherein

R^6 and R^7 are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^1 and R^5 are members independently selected from OR^9, NR^9R^{10}, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl

wherein

R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and $C(O)R^{11}$

in which

R^{11} is selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl,

substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl;

R^2 is a member selected from OH and an activating moiety;

R^3 is a member selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl; and

R^4 is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and $C(O)R^{12}$

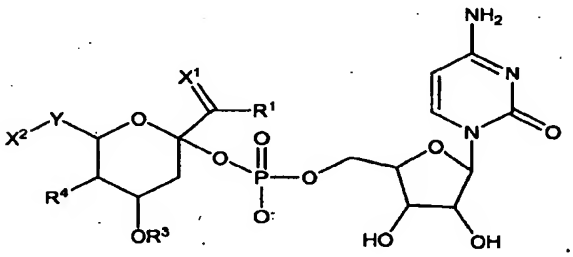
in which

R^{12} is a member selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl and $NR^{13}R^{14}$

wherein

R^{13} and R^{14} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl.

8. The compound according to claim 7 having the formula:



9. The compound according to claim 7, wherein X^2 is $N(R^6)$-L-$(m\text{-}PEG)_s$ wherein

L is a linker moiety which is a member selected from an amino acid residue and a peptidyl residue; and

s is an integer from 1 to 3.

10. A method of forming a covalent conjugate between a modified saccharyl fragment and a glycosylated or non-glycosylated peptide, said method comprising:

enzymatically transferring a modified saccharyl fragment from an activated modified saccharyl fragment to an acceptor moiety on said peptide.

11. The method according to claim 10, wherein said modified saccharyl fragment is covalently attached to a glycosyl residue covalently attached to said peptide.

12. The method according to claim 10, wherein said modified saccharyl fragment is covalently attached to an amino acid residue of said peptide

13. The method of claim 10, wherein said enzyme is a glycosyltransferase which is a member selected from the group consisting of sialyl transferases, trans-sialidases, galactosyltransferases, glucosyltransferases, GalNAc transferase, GlcNAc transferase, fucosyl transferases, and mannosyltransferases.

14. The method of claim 13, wherein said glycosyltransferase is recombinant.

15. The method according to claim 10, wherein said method is performed in a cell-free environment.

16. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a conjugate comprising a modified saccharyl fragment covalently linked to a glycosylated or non-glycosylated peptide.

17. A composition for forming a conjugate between a peptide and a modified saccharyl fragment, said composition comprising: a mixture of an activated modified saccharyl fragment, an enzyme for which said saccharyl fragment is a substrate, and a peptide acceptor substrate, wherein said modified saccharyl fragment has covalently attached thereto a member selected from water-soluble polymers, therapeutic moieties and biomolecules.

GLYCOCONJUGATION USING SACCHARYL FRAGMENTS

ABSTRACT OF THE DISCLOSURE

The present invention provides conjugates between a substrate, e.g., peptide, glycopeptide, lipid, etc., and a modified saccharyl fragment bearing a modifying group such as a water-soluble polymer, therapeutic moiety or a biomolecule. The conjugates are linked via the enzymatic conversion of the activated modified saccharyl fragment into a glycosyl linking group that is interposed between and covalently attached to the substrate and the modifying group. The conjugates are formed from substrates by the action of a sugar transferring enzyme, e.g., a glycosyltransferase. For example, when the substrate is a peptide, the enzyme conjugates a modified saccharyl fragment moiety onto either an amino acid or glycosyl residue of the peptide. Also provided are pharmaceutical formulations that include the conjugates. Methods for preparing the conjugates are also within the scope of the invention.

FIGURE 1A

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
At1g08280	*Arabidopsis thaliana*	n.d.	AC011438 AAF18241.1 BT004583 AAO42829.1 NC_003070 NP_172305.1	Q84W00 Q9SGD2	
At1g08660/F22O13.14	*Arabidopsis thaliana*	n.d.	AC003981 AAF99778.1 AY064135 AAL36042.1 AY124807 AAM70516.1 NC_003070 NP_172342.1 NM_180609 NP_850940.1	Q8VZJ0 Q9FRR9	
At3g48820/T21J18_90	*Arabidopsis thaliana*	n.d.	AY080589 AAL85966.1 AY133816 AAM91750.1 AL132963 CAB87910.1 NM_114741 NP_190451.1	Q8RY00 Q9M301	
α-2,3-sialyltransferase (ST3GAL-IV)	*Bos taurus*	n.d.	AJ584673 CAE48298.1		
α-2,3-sialyltransferase (St3Gal-V)	*Bos taurus*	n.d.	AJ585768 CAE51392.1		
α-2,6-sialyltransferase (Siat7b)	*Bos taurus*	n.d.	AJ620651 CAF05850.1		
α-2,8-sialyltransferase (SIAT8A)	*Bos taurus*	2.4.99.8	AJ699418 CAG27880.1		
α-2,8-sialyltransferase (Siat8D)	*Bos taurus*	n.d.	AJ699421 CAG27883.1		
α-2,8-sialyltransferase ST8Sia-III (Siat8C)	*Bos taurus*	n.d.	AJ704563 CAG28696.1		
CMP α-2,6-sialyltransferase (ST6Gal I)	*Bos taurus*	2.4.99.1	Y15111 CAA75385.1 NM_177517 NP_803483.1	O18974	
sialyltransferase 8 (fragment)	*Bos taurus*	n.d.	AF450088 AAL47018.1	Q8WN13	
sialyltransferase ST3Gal-II (Siat4B)	*Bos taurus*	n.d.	AJ748841 CAG44450.1		
sialyltransferase ST3Gal-III (Siat6)	*Bos taurus*	n.d.	AJ748842 CAG44451.1		
sialyltransferase ST3Gal-VI (Siat10)	*Bos taurus*	n.d.	AJ748843 CAG44452.1		
ST3Gal I	*Bos taurus*	n.d.	AJ305086 CAC24698.1	Q9BEG4	
St6GalNAc-VI	*Bos taurus*	n.d.	AJ620949 CAF06586.1		
CDS4	*Branchiostoma floridae*	n.d.	AF391289 AAM18873.1	Q8T771	
polysialyltransferase (PST) (fragment) ST8Sia IV	*Cercopithecus aethiops*	2.4.99.-	AF210729 AAF17105.1	Q9TT09	
polysialyltransferase (STX) (fragment) ST8Sia II	*Cercopithecus aethiops*	2.4.99.-	AF210318 AAF17104.1	Q9TT10	
α-2,3-sialyltransferase ST3Gal I (Siat4)	*Ciona intestinalis*	n.d.	AJ626815 CAF25173.1		
α-2,3-sialyltransferase ST3Gal I (Siat4)	*Ciona savignyi*	n.d.	AJ626814 CAF25172.1		
α-2,8-polysialyltransferase ST8Sia IV	*Cricetulus griseus*	2.4.99.-	-AAE28634 Z46801 CAA86822.1	Q64690	
Gal β-1,3/4-GlcNAc α-2,3-sialyltransferase St3Gal I	*Cricetulus griseus*	n.d.	AY266675 AAP22942.1	Q80WL0	
Gal β-1,3/4-GlcNAc α-2,3-sialyltransferase St3Gal II (fragment)	*Cricetulus griseus*	n.d.	AY266676 AAP22943.1	Q80WK9	
α-2,3-sialyltransferase ST3Gal I (Siat4)	*Danio rerio*	n.d.	AJ783740 CAH04017.1		
α-2,3-sialyltransferase ST3Gal II (Siat5)	*Danio rerio*	n.d.	AJ783741 CAH04018.1		
α-2,3-sialyltransferase ST3Gal III (Siat6)	*Danio rerio*	n.d.	AJ626821 CAF25179.1		
α-2,3-sialyltransferase ST3Gal IV (Siat4c)	*Danio rerio*	n.d.	AJ744809 CAG32845.1		

FIGURE 1B

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
α-2,3-sialyltransferase ST3Gal V-r (Siat5-related)	*Danio rerio*	n.d.	AJ783742 CAH04019.1		
α-2,6-sialyltransferase ST6Gal I (Siat1)	*Danio rerio*	n.d.	AJ744801 CAG32837.1		
α-2,6-sialyltransferase ST6GalNAc II (Siat7B)	*Danio rerio*	n.d.	AJ634459 CAG25680.1		
α-2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)	*Danio rerio*	n.d.	AJ646874 CAG26703.1		
α-2,6-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)	*Danio rerio*	n.d.	AJ646883 CAG26712.1		
α-2,8-sialyltransferase ST8Sia I (Siat 8A) (fragment)	*Danio rerio*	n.d.	AJ715535 CAG29374.1		
α-2,8-sialyltransferase ST8Sia III (Siat 8C) (fragment)	*Danio rerio*	n.d.	AJ715543 CAG29382.1		
α-2,8-sialyltransferase ST8Sia IV (Siat 8D) (fragment)	*Danio rerio*	n.d.	AJ715545 CAG29384.1		
α-2,8-sialyltransferase ST8Sia V (Siat 8E) (fragment)	*Danio rerio*	n.d.	AJ715546 CAG29385.1		
α-2,8-sialyltransferase ST8Sia VI (Siat 8F) (fragment)	*Danio rerio*	n.d.	AJ715551 CAG29390.1		
β-galactosamide α-2,6-sialyltransferase II (ST6Gal II)	*Danio rerio*	n.d.	AJ627627 CAF29495.1		
N-glycan α-2,8-sialyltransferase	*Danio rerio*	n.d.	BC050483 AAH50483.1 AY055462 AAL17875.1 NM_153662 NP_705948.1	Q7ZU51 Q8QH83	
ST3Gal III-related (siat6r)	*Danio rerio*	n.d.	BC053179 AAH53179.1 AJ626820 CAF25178.1 NM_200355 NP_956649.1	Q7T3B9	
St3Gal-V	*Danio rerio*	n.d.	AJ619960 CAF04061.1		
st6GalNAc-VI	*Danio rerio*	n.d.	BC060932 AAH60932.1 AJ620947 CAF06584.1		
α-2,6-sialyltransferase (CG4871) ST6Gal I	*Drosophila melanogaster*	2.4.99.1	AE003465 AAF47256.1 AF218237 AAG13185.1 AF397532 AAK92126.1 AE003465 AAM70791.1 NM_079129 NP_523853.1 NM_166684 NP_726474.1	Q9GU23 Q9W121	
α-2,3-sialyltransferase (ST3Gal-VI)	*Gallus gallus*	n.d.	AJ585767 CAE51391.1 AJ627204 CAF25503.1		
α-2,3-sialyltransferase ST3Gal I	*Gallus gallus*	2.4.99.4	X80503 CAA56666.1 NM_205217 NP_990548.1	Q11200	
α-2,3-sialyltransferase ST3Gal IV (fragment)	*Gallus gallus*	2.4.99.-	AF035250 AAC14163.1	O73724	
α-2,3-sialytransferase (ST3GAL-II)	*Gallus gallus*	n.d.	AJ585761 CAE51385.2		
α-2,6-sialyltransferase (Siat7b)	*Gallus gallus*	n.d.	AJ620653 CAF05852.1		
α-2,6-sialyltransferase ST6Gal I	*Gallus gallus*	2.4.99.1	X75558 CAA53235.1 NM_205241 NP_990572.1	Q92182	
α-2,6-sialyltransferase ST6GalNAc I	*Gallus gallus*	2.4.99.3	-AAE68028.1 -AAE68029.1 X74946 CAA52902.1 NM_205240 NP_990571.1	Q92183	
α-2,6-sialyltransferase ST6GalNAc II	*Gallus gallus*	2.4.99.-	X77775 AAE68030.1 NM_205233 CAA54813.1 NP_990564.1	Q92184	
α-2,6-sialyltransferase ST6GalNAc III (SIAT7C)	*Gallus gallus*	n.d.	AJ634455 CAG25677.1		

FIGURE 1C

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
(fragment)					
α-2,6-sialyltransferase ST6GalNAc V (SIAT7E) (fragment)	*Gallus gallus*	n.d.	AJ646877 **CAG26706.1**		
α-2,8-sialyltransferase (GD3 Synthase) ST8Sia I	*Gallus gallus*	2.4.99.-	U73176 AAC28888.1	**P79783**	
α-2,8-sialyltransferase (SIAT8B)	*Gallus gallus*	n.d.	AJ699419 **CAG27881.1**		
α-2,8-sialyltransferase (SIAT8C)	*Gallus gallus*	n.d.	AJ699420 **CAG27882.1**		
α-2,8-sialyltransferase (SIAT8F)	*Gallus gallus*	n.d.	AJ699424 **CAG27886.1**		
α-2,8-syalyltransferase ST8Siα-V (SIAT8C)	*Gallus gallus*	n.d.	AJ704564 **CAG28697.1**		
β-galactosamide α-2,6-sialyltransferase II (ST6Gal II)	*Gallus gallus*	n.d.	AJ627629 **CAF29497.1**		
GM3 synthase (SIAT9)	*Gallus gallus*	2.4.99.9	AY515255 **AAS83519.1**		
polysialyltransferase ST8Sia IV	*Gallus gallus*	2.4.99.-	AF008194 AAB95120.1	**O42399**	
α-2,3-sialyltransferase ST3Gal I	*Homo sapiens*	2.4.99.4	L29555 AAA36612.1	Q11201	
			AF059321 AAC17874.1	O60677	
			L13972 AAC37574.1	Q9UN51	
			AF155238 AAD39238.1		
			AF186191 **AAG29876.1**		
			BC018357 AAH18357.1		
			NM_003033 NP_003024.1		
			NM_173344 NP_775479.1		
α-2,3-sialyltransferase ST3Gal II	*Homo sapiens*	2.4.99.4	U63090 AAB40389.1	Q16842	
			BC036777 **AAH36777.1**	O00654	
			X96667 CAA65447.1		
			NM_006927 NP_008858.1		
α-2,3-sialyltransferase ST3Gal III (SiaT6)	*Homo sapiens*	2.4.99.6	L23768 AAA35778.1	Q11203	
			BC050380 AAH50380.1	Q86UR6	
			AF425851 AAO13859.1	Q86UR7	
			AF425852 AAO13860.1	Q86UR8	
			AF425853 AAO13861.1	Q86UR9	
			AF425854 AAO13862.1	Q86US0	
			AF425855 AAO13863.1	Q86US1	
			AF425856 AAO13864.1	Q86US2	
			AF425857 AAO13865.1	Q8IX43	
			AF425858 AAO13866.1	Q8IX44	
			AF425859 AAO13867.1	Q8IX45	
			AF425860 AAO13868.1	Q8IX46	
			AF425861 AAO13869.1	Q8IX47	
			AF425862 **AAO13870.1**	Q8IX48	
			AF425863 AAO13871.1	Q8IX49	
			AF425864 AAO13872.1	Q8IX50	
			AF425865 AAO13873.1	Q8IX51	
			AF425866 AAO13874.1	Q8IX52	
			AF425867 AAO13875.1	Q8IX53	
			AY167992 AAO38806.1	Q8IX54	
			AY167993 AAO38807.1	Q8IX55	
			AY167994 AAO38808.1	Q8IX56	
			AY167995 AAO38809.1	Q8IX57	
			AY167996 AAO38810.1	Q8IX58	
			AY167997 AAO38811.1		
			AY167998 AAO38812.1		
			NM_006279 NP_006270.1		
			NM_174964 NP_777624.1		
			NM_174965 NP_777625.1		
			NM_174966 NP_777626.1		
			NM_174967 NP_777627.1		
			NM_174969 NP_777629.1		
			NM_174970 NP_777630.1		
			NM_174972 NP_777632.1		

FIGURE 1D

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
α-2,3-sialyltransferase ST3Gal IV	*Homo sapiens*	2.4.99.-	L23767 AAA16460.1 AF035249 AAC14162.1 BC010645 **AAH10645.1** AY040826 AAK93790.1 AF516602 AAM66431.1 AF516603 AAM66432.1 AF516604 AAM66433.1 AF525084 AAM81378.1 X74570 CAA52662.1 CR456858 CAG33139.1 NM_006278 NP_006269.1	Q11206 O60497 Q96QQ9 Q8N6A6 Q8N6A7 Q8NFD3 Q8NFG7	
α-2,3-sialyltransferase ST3Gal VI	*Homo sapiens*	2.4.99.4	AF119391 **AAD39131.1** BC023312 AAH23312.1 AB022918 BAA77609.1 AX877828 CAE89895.1 AX886023 CAF00161.1 NM_006100 NP_006091.1	Q9Y274	
α-2,6-sialyltransferase (ST6Gal II ; KIAA1877)	*Homo sapiens*	n.d.	BC008680 AAH08680.1 AB058780 **BAB47506.1** AB059555 BAC24793.1 AJ512141 CAD54408.1 AX795193 CAE48260.1 AX795193 CAE48261.1 NM_032528 NP_115917.1	Q86Y44 Q8IUG7 Q96HE4 Q96JF0	
α-2,6-sialyltransferase (ST6GALNAC III)	*Homo sapiens*	n.d.	BC059363 AAH59363.1 AY358540 AAQ88904.1 AK091215 BAC03611.1 AJ507291 **CAD45371.1** NM_152996 NP_694541.1	Q8N259 Q8NDV1	
α-2,6-sialyltransferase (ST6GalNAc V)	*Homo sapiens*	n.d.	BC001201 **AAH01201.1** AK056241 BAB71127.1 AL035409 CAB72344.1 AJ507292 CAD45372.1 NM_030965 NP_112227.1	Q9BVH7	
α-2,6-sialyltransferase (SThM) ST6GalNAc II	*Homo sapiens*	2.4.99.-	U14550 AAA52228.1 BC040455 **AAH40455.1** AJ251053 CAB61434.1 NM_006456 NP_006447.1	Q9UJ37 Q12971	
α-2,6-sialyltransferase ST6Gal I	*Homo sapiens*	2.4.99.1	BC031476 AAH31476.1 BC040009 AAH40009.1 A17362 **CAA01327.1** A23699 CAA01686.1 X17247 CAA35111.1 X54363 CAA38246.1 X62822 CAA44634.1 NM_003032 NP_003023.1 NM_173216 NP_775323.1	P15907	
α-2,6-sialyltransferase ST6GalNAc I	*Homo sapiens*	2.4.99.3	BC022462 AAH22462.1 AY096001 AAM22800.1 AY358918 AAQ89277.1 AK000113 BAA90953.1 Y11339 **CAA72179.2** NM_018414 NP_060884.1	Q8TBJ6 Q9NSC7 Q9NXQ7	
α-2,8-polysialyltransferase ST8Sia IV	*Homo sapiens*	2.4.99.-	L41680 AAC41775.1 BC027866 AAH27866.1 BC053657 **AAH53657.1** NM_005668 NP_005659.1	Q8N1F4 Q92187 Q92693	
α-2,8-sialyltransferase (GD3 synthase) ST8Sia I	*Homo sapiens*	2.4.99.8	L32867 AAA62366.1 L43494 **AAC37586.1** BC046158 AAH46158.1 AAQ53140.1 AY569975 AAS75783.1 D26360 BAA05391.1 X77922 CAA54891.1 NM_003034 NP_003025.1	Q86X71 Q92185 Q93064	
α-2,8-sialyltransferase	*Homo sapiens*	2.4.99.-	L29556 AAA36613.1	Q92186	

FIGURE 1E

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
ST8Sia II			U82762 AAB51242.1 U33551 AAC24458.1 BC069584 AAH69584.1 NM_006011 NP_006002.1	Q92470 Q92746	
α-2,8-sialyltransferase ST8Sia III	*Homo sapiens*	2.4.99.-	AF004668 AAB87642.1 AF003092 AAC15901.2 NM_015879 NP_056963.1	O43173 Q9NS41	
α-2,8-sialyltransferase ST8Sia V	*Homo sapiens*	2.4.99.-	U91641 AAC51727.1 CR457037 CAG33318.1 NM_013305 NP_037437.1	O15466	
ENSP00000020221 (fragment)		n.d.	AC023295-		
lactosylceramide α-2,3-sialyltransferase (ST3Gal V)	*Homo sapiens*	2.4.99.9	AF105026 AAD14634.1 AF119415 AAF66146.1 BC065936 AAH65936.1 AY152815 AAO16866.1 AAP65066 AAP65066.1 AY359105 AAQ89463.1 AB018356 BAA33950.1 AX876536 CAE89320.1 NM_003896 NP_003887.2	Q9UNP4 O94902	
N-acetylgalactosaminide α-2,6-sialyltransferase (ST6GalNAc VI)	*Homo sapiens*	2.4.99.-	BC006564 AAH06564.1 BC007802 AAH07802.1 BC016299 AAH16299.1 AY358672 AAQ89035.1 AB035173 BAA87035.1 AK023900 BAB14715.1 AJ507293 CAD45373.1 AX880950 CAE91145.1 CR457318 CAG33599.1 NM_013443 NP_038471.2	Q969X2 Q9H8A2 Q9ULB8	
N-acetylgalactosaminide α-2,6-sialyltransferase IV (ST6GalNAc IV)	*Homo sapiens*	2.4.99.-	AF127142 AAF00102.1 BC036705 AAH36705.1 - AAP63349.1 AB035172 BAA87034.1 AK000600 BAA91281.1 Y17461 CAB44354.1 AJ271734 CAC07404.1 AX061620 CAC24981.1 AX068265 CAC27250.1 AX969252 CAF14360.1 NM_014403 NP_055218.3 NM_175039 NP_778204.1	Q9H4F1 Q9NWU6 Q9UKU1 Q9ULB9 Q9Y3G3 Q9Y3G4	
ST8SIA-VI (fragment)	*Homo sapiens*	n.d.	AJ621583 CAF21722.1 XM_291725 XP_291725.2		
unnamed protein product	*Homo sapiens*	n.d.	AK021929 BAB13940.1 AX881696 CAE91353.1	Q9HAA9	
Gal β-1,3/4-GlcNAc α-2,3-sialyltransferase (ST3Gal III)	*Mesocricetus auratus*	2.4.99.6	AJ245699 CAB53394.1	Q9QXF6	

FIGURE 1F

Protein		Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
Gal β-1,3/4-GlcNAc α-2,3-sialyltransferase (ST3Gal IV)		*Mesocricetus auratus*	2.4.99.6	AJ245700 **CAB53395.1**	Q9QXF5	
GD3 synthase (fragment) ST8Sia I		*Mesocricetus auratus*	n.d.	AF141657 **AAD33879.1**	Q9WUL1	
polysialyltransferase (ST8Sia IV)		*Mesocricetus auratus*	2.4.99.-	AJ245701 **CAB53396.1**	Q9QXF4	
α-2,3-sialyltransferase ST3Gal I	*St3gal1*	*Mus musculus*	2.4.99.4	AF214028 AAF60973.1 AK031344 BAC27356.1 AK078469 BAC37290.1 X73523 CAA51919.1 NM_009177 NP_033203.1	**P54751** **Q11202** Q9JL30	
α-2,3-sialyltransferase ST3Gal II	*St3gal2*	*Mus musculus*	2.4.99.4	BC015264 **AAH15264.1** BC066064 AAH66064.1 AK034554 BAC28752.1 AK034863 BAC28859.1 AK053827 BAC35543.1 X76989 CAA54294.1 NM_009179 NP_033205.1 NM_178048 NP_835149.1	Q11204 Q8BPL0 Q8BSA0 Q8BSE9 Q91WH6	
α-2,3-sialyltransferase ST3Gal III	*St3gal3*	*Mus musculus*	2.4.99.-	BC006710 AAH06710.1 AK005053 **BAB23779.1** AK013016 BAB28598.1 X84234 CAA59013.1 NM_009176 NP_033202.2	P97325 Q922X5 Q9CZ48 Q9DBB6	
α-2,3-sialyltransferase ST3Gal IV	*St3gal4*	*Mus musculus*	2.4.99.4	BC011121 AAH11121.1 BC050773 **AAH50773.1** D28941 BAA06068.1 AK008543 BAB25732.1 AB061305 BAB47508.1 X95809 CAA65076.1 NM_009178 NP_033204.2	P97354 Q61325 Q91Y74 Q921R5 Q9CVE8	
α-2,3-sialyltransferase ST3Gal VI	*St3gal6*	*Mus musculus*	2.4.99.4	AF119390 AAD39130.1 BC052338 AAH52338.1 AB063326 **BAB79494.1** AK033562 BAC28360.1 AK041173 BAC30851.1 NM_018784 NP_061254	Q80UR7 Q8BLV1 Q8VIB3 Q9WVG2	
α-2,6-sialyltransferase ST6GalNAc II	*St6galnac2*	*Mus musculus*	2.4.99.-	NM_009180 6677963 BC010208 AAH10208.1 AB027198 BAB00637.1 AK004613 BAB23410.1 X93999 CAA63821.1 X94000 CAA63822.1 NM_009180 NP_033206.2	**P70277** Q9DC24 Q9JJM5	
α-2,6-sialyltransferase ST6Gal I	*St6gal1*	*Mus musculus*	2.4.99.1	-AAE68031.1 BC027833 AAH27833.1 D16106 BAA03680.1 AK034768 BAC28828.1 AK084124 BAC39120.1 NM_145933 NP_666045.1	**Q64685** Q8BM62 Q8K1L1	
α-2,6-sialyltransferase ST6Gal II	*St6gal2*	*Mus musculus*	n.d.	AK082566 BAC38534.1 AB095093 **BAC87752.1** AK129462 BAC98272.1 NM_172829 NP_766417.1	Q8BUU4	
α-2,6-sialyltransferase ST6GalNAc I	*St6galnac1*	*Mus musculus*	2.4.99.3	Y11274 **CAA72137.1** NM_011371 NP_035501.1	Q9QZ39 Q9JJP5	
α-2,6-sialyltransferase ST6GalNAc III	*St6galnac3*	*Mus musculus*	n.d.	BC058387 AAH58387.1 AK034804 BAC28836.1 Y11342 CAA72181.2 Y11343 **CAB95031.1** NM_011372 NP_035502	Q9WUV2 Q9JHP5	
α-2,6-sialyltransferase ST6GalNAc IV	*St6galnac4*	*Mus musculus*	2.4.99.7	BC056451 AAH56451.1 AK085730 BAC39523.1 AJ007310 CAA07446.1 Y15779 CAB43507.1	Q8C3J2 Q9JHP2 Q9R2B6 O88725	

FIGURE 1G

Protein		Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
				Y15780 **CAB43514.1**	Q9JHP0	
				Y19055 CAB93946.1	Q9QUP9	
				Y19057 CAB93948.1	Q9R2B5	
				NM_011373 NP_035503.1		
α-2,8-sialyltransferase (GD3 synthase) ST8Sia I	*St8sia1*	*Mus musculus*	2.4.99.8	L38677 AAA91869.1	Q64468	
				BC024821 **AAH24821.1**	Q64687	
				AK046188 BAC32625.1	Q8BL76	
				AK052444 BAC34994.1	Q8BWI0	
				X84235 CAA59014.1	Q8K1C1	
				AJ401102 CAC20706.1	Q9EPK0	
				NM_011374 NP_035504.1		
α-2,8-sialyltransferase (ST8Sia VI)	*St8sia6*	*Mus musculus*	n.d.	AB059554 **BAC01265.1**	Q8BI43	
				AK085105 BAC39367.1	Q8K4T1	
				NM_145838 NP_665837.1		
α-2,8-sialyltransferase ST8Sia II	*St8sia2*	*Mus musculus*	2.4.99.-	X83562 CAA58548.1	**O35696**	
				X99646 CAA67965.1		
				X99647 CAA67965.1		
				X99648 CAA67965.1		
				X99649 CAA67965.1		
				X99650 CAA67965.1		
				X99651 CAA67965.1		
				NM_009181 NP_033207.1		
α-2,8-sialyltransferase ST8Sia IV	*St8sia4*	*Mus musculus*	2.4.99.8	BC060112 AAH60112.1	Q64692	
				AK003690 BAB22941.1	Q8BY70	
				AK041723 BAC31044.1		
				AJ223958 **CAA11685.1**		
				X86000 CAA59992.1		
				Y09484 CAA70692.1		
				NM_009183 NP_033209.1		
α-2,8-sialyltransferase ST8Sia V	*St8sia5*	*Mus musculus*	2.4.99.-	BC034855 AAH34855.1	P70126	
				AK078670 BAC37354.1	P70127	
				X98014 **CAA66642.1**	P70128	
				X98014 CAA66643.1	Q8BJW0	
				X98014 CAA66644.1	Q8JZQ3	
				NM_013666 NP_038694.1		
				NM_153124 NP_694764.1		
				NM_177416 NP_803135.1		
α-2,8-sialytransferase ST8Sia III	*St8sia3*	*Mus musculus*	2.4.99.-	BC075645 AAH75645.1	**Q64689**	
				AK015874 BAB30012.1	Q9CUJ6	
				X80502 CAA56665.1		
				NM_009182 NP_033208.1		
GD1 synthase (ST6GalNAc V)	*St6galnac5*	*Mus musculus*	n.d.	BC055737 **AAH55737.1**	Q8CAM7	
				AB030836 BAA85747.1	Q8CBX1	
				AB028840 BAA89292.1	Q9QYJ1	
				AK034387 BAC28693.1	Q9R0K6	
				AK038434 BAC29997.1		
				AK042683 BAC31331.1		
				NM_012028 NP_036158.2		
GM3 synthase (α-2,3-sialyltransferase) ST3Gal V	*St3gal5*	*Mus musculus*	2.4.99.9	AF119416 **AAF66147.1**	O88829	
				AAP65063.1	Q9CZ65	
				AB018048 BAA33491.1	Q9QWF9	
				AB013302 BAA76467.1		
				AK012961 BAB28571.1		
				Y15003 CAA75235.1		
				NM_011375 NP_035505.1		
N-acetylgalactosaminide α-2,6-sialyltransferase (ST6GalNAc VI)	*St6galnac6*	*Mus musculus*	2.4.99.-	BC036985 **AAH36985.1**	Q8CDC3	
				AB035174 BAA87036.1	Q8JZW3	
				AB035123 BAA95940.1	Q9JM95	
				AK030648 BAC27064.1	Q9R0G9	
				NM_016973 NP_058669.1		
M138L		*Myxoma virus*	n.d.	U46578 AAD00069.1		
				AF170726 AAE61323.1		
				NC_001132 AAE61326.1		
				AAF15026.1		
				NP_051852.1		
α-2,3-sialyltransferase		*Oncorhynchus*	n.d.	AJ585760 **CAE51384.1**		

FIGURE 1H

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
(St3Gal-I)	*mykiss*				
α-2,6-sialyltransferase (Siat1)	*Oncorhynchus mykiss*	n.d.	AJ620649 CAF05848.1		
α-2,8-polysialyltransferase IV (ST8Sia IV)	*Oncorhynchus mykiss*	n.d.	AB094402 BAC77411.1	Q7T2X5	
GalNAc α-2,6-sialyltransferase (RtST6GalNAc)	*Oncorhynchus mykiss*	n.d.	AB097943 BAC77520.1	Q7T2X4	
α-2,3-sialyltransferase ST3Gal IV	*Oryctolagus cuniculus*	2.4.99.-	AF121967 AAF28871.1	Q9N257	
OJ1217_F02.7	*Oryza sativa (japonica cultivar-group)*	n.d.	AP004084 BAD07616.1		
OSJNBa0043L24.2 or OSJNBb0002J11.9	*Oryza sativa (japonica cultivar-group)*	n.d.	AL731626 CAD41185.1 AL662969 CAE04714.1		
P0683f02.18 or P0489B03.1	*Oryza sativa (japonica cultivar-group)*	n.d.	AP003289 BAB63715.1 AP003794 BAB90552.1		
α-2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)	*Oryzias latipes*	n.d.	AJ646876 CAG26705.1		
α-2,3-sialyltransferase ST3Gal I (Siat4)	*Pan troglodytes*	n.d.	AJ744803 CAG32839.1		
α-2,3-sialyltransferase ST3Gal II (Siat5)	*Pan troglodytes*	n.d.	AJ744804 CAG32840.1		
α-2,3-sialyltransferase ST3Gal III (Siat6)	*Pan troglodytes*	n.d.	AJ626819 CAF25177.1		
α-2,3-sialyltransferase ST3Gal IV (Siat4c)	*Pan troglodytes*	n.d.	AJ626824 CAF25182.1		
α-2,3-sialyltransferase ST3Gal VI (Siat10)	*Pan troglodytes*	n.d.	AJ744808 CAG32844.1		
α-2,6-sialyltransferase (Sia7A)	*Pan troglodytes*	n.d.	AJ748740 CAG38615.1		
α-2,6-sialyltransferase (Sia7B)	*Pan troglodytes*	n.d.	AJ748741 CAG38616.1		
α-2,6-sialyltransferase ST6GalNAc III (Siat7C)	*Pan troglodytes*	n.d.	AJ634454 CAG25676.1		
α-2,6-sialyltransferase ST6GalNAc IV (Siat7D) (fragment)	*Pan troglodytes*	n.d.	AJ646870 CAG26699.1		
α-2,6-sialyltransferase ST6GalNAc V (Siat7E)	*Pan troglodytes*	n.d.	AJ646875 CAG26704.1		
α-2,6-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)	*Pan troglodytes*	n.d.	AJ646882 CAG26711.1		
α-2,8-sialyltransferase 8A (Siat8A)	*Pan troglodytes*	2.4.99.8	AJ697658 CAG26896.1		
α-2,8-sialyltransferase 8B (Siat8B)	*Pan troglodytes*	n.d.	AJ697659 CAG26897.1		
α-2,8-sialyltransferase 8C (Siat8C)	*Pan troglodytes*	n.d.	AJ697660 CAG26898.1		
α-2,8-sialyltransferase 8D (Siat8D)	*Pan troglodytes*	n.d.	AJ697661 CAG26899.1		
α-2,8-sialyltransferase 8E (Siat8E)	*Pan troglodytes*	n.d.	AJ697662 CAG26900.1		
α-2,8-sialyltransferase 8F (Siat8F)	*Pan troglodytes*	n.d.	AJ697663 CAG26901.1		
β-galactosamide α-2,6-sialyltransferase I (ST6Gal I; Siat1)	*Pan troglodytes*	2.4.99.1	AJ627624 CAF29492.1		
β-galactosamide α-2,6-sialyltransferase II (ST6Gal II)	*Pan troglodytes*	n.d.	AJ627625 CAF29493.1		
GM3 synthase ST3Gal V	*Pan troglodytes*	n.d.	AJ744807 CAG32843.1		

FIGURE 1I

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
(Siat9)					
S138L	*Rabbit fibroma virus Kasza*	n.d.	NC_001266 NP_052025		
α-2,3-sialyltransferase ST3Gal III	*Rattus norvegicus*	2.4.99.6	M97754 AAA42146.1 NM_031697 NP_113885.1	Q02734	
α-2,3-sialyltransferase ST3Gal IV (Siat4c)	*Rattus norvegicus*	n.d.	AJ626825 CAF25183.1		
α-2,3-sialyltransferase ST3Gal VI	*Rattus norvegicus*	n.d.	AJ626743 CAF25053.1		
α-2,6-sialyltransferase ST3Gal II	*Rattus norvegicus*	2.4.99.-	X76988 CAA54293.1 NM_031695 NP_113883.1	Q11205	
α-2,6-sialyltransferase ST6Gal I	*Rattus norvegicus*	2.4.99.1	M18769 AAA41196.1 M83143 AAB07233.1	P13721	
α-2,6-sialyltransferase ST6GalNAc I (Siat7A)	*Rattus norvegicus*	n.d.	AJ634458 CAG25684.1		
α-2,6-sialyltransferase ST6GalNAc II (Siat7B)	*Rattus norvegicus*	n.d.	AJ634457 CAG25679.1		
α-2,6-sialyltransferase ST6GalNAc III	*Rattus norvegicus*	2.4.99.-	L29554 AAC42086.1 BC072501 AAH72501.1 NM_019123 NP_061996.1	Q64686	
α-2,6-sialyltransferase ST6GalNAc IV (Siat7D) (fragment)	*Rattus norvegicus*	n.d.	AJ646871 CAG26700.1		
α-2,6-sialyltransferase ST6GalNAc V (Siat7E)	*Rattus norvegicus*	n.d.	AJ646872 CAG26701.1		
α-2,6-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)	*Rattus norvegicus*	n.d.	AJ646881 CAG26710.1		
α-2,8-sialyltransferase (GD3 synthase) ST8Sia I	*Rattus norvegicus*	2.4.99.-	U53883 AAC27541.1 D45255 BAA08213.1	P70554 P97713	
α-2,8-sialyltransferase (SIAT8E)	*Rattus norvegicus*	n.d.	AJ699422 CAG27884.1		
α-2,8-sialyltransferase (SIAT8F)	*Rattus norvegicus*	n.d.	AJ699423 CAG27885.1		
α-2,8-sialyltransferase ST8Sia II	*Rattus norvegicus*	2.4.99.-	L13445 AAA42147.1 NM_057156 NP_476497.1	Q07977 Q64688	
α-2,8-sialyltransferase ST8Sia III	*Rattus norvegicus*	2.4.99.-	U55938 AAB50061.1 NM_013029 NP_037161.1	P97877	
α-2,8-sialyltransferase ST8Sia IV	*Rattus norvegicus*	2.4.99.-	U90215 AAB49989.1	O08563	
β-galactosamide α-2,6-sialyltransferase II (ST6Gal II)	*Rattus norvegicus*	n.d.	AJ627626 CAF29494.1		
GM3 synthase ST3Gal V	*Rattus norvegicus*	n.d.	AB018049 BAA33492.1 NM_031337 NP_112627.1	O88830	
sialyltransferase ST3Gal-I (Siat4A)	*Rattus norvegicus*	n.d.	AJ748840 CAG44449.1		
α-2,3-sialyltransferase (St3Gal-II)	*Silurana tropicalis*	n.d.	AJ585763 CAE51387.1		
α-2,6-sialyltransferase (Siat7b)	*Silurana tropicalis*	n.d.	AJ620650 CAF05849.1		
α-2,6-sialyltransferase (St6galnac)	*Strongylocentrotus purpuratus*	n.d.	AJ699425 CAG27887.1		
α-2,3-sialyltransferase (ST3GAL-III)	*Sus scrofa*	n.d.	AJ585765 CAE51389.1		
α-2,3-sialyltransferase (ST3GAL-IV)	*Sus scrofa*	n.d.	AJ584674 CAE48299.1		
α-2,3-sialyltransferase ST3Gal I	*Sus scrofa*	2.4.99.4	M97753 AAA31125.1	Q02745	
α-2,6-sialyltransferase (fragment) ST6Gal I	*Sus scrofa*	2.4.99.1	AF136746 AAD33059.1	Q9XSG8	
β-galactosamide α-2,6-sialyltransferase (ST6GalNAc-V)	*Sus scrofa*	n.d.	AJ620948 CAF06585.2		
sialyltransferase (fragment) ST6Gal I	*sus scrofa*	n.d.	AF041031 AAC15633.1	O62717	

FIGURE 1J

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
ST6GALNAC-V	*Sus scrofa*	n.d.	AJ620948 CAF06585.1		
α-2,3-sialyltransferase (Siat5-r)	*Takifugu rubripes*	n.d.	AJ744805 CAG32841.1		
α-2,3-sialyltransferase ST3Gal I (Siat4)	*Takifugu rubripes*	n.d.	AJ626816 CAF25174.1		
α-2,3-sialyltransferase ST3Gal II (Siat5) (fragment)	*Takifugu rubripes*	n.d.	AJ626817 CAF25175.1		
α-2,3-sialyltransferase ST3Gal III (Siat6)	*Takifugu rubripes*	n.d.	AJ626818 CAF25176.1		
α-2,6-sialyltransferase ST6Gal I (Siat1)	*Takifugu rubripes*	n.d.	AJ744800 CAG32836.1		
α-2,6-sialyltransferase ST6GalNAc II (Siat7B)	*Takifugu rubripes*	n.d.	AJ634460 CAG25681.1		
α-2,6-sialyltransferase ST6GalNAc II B (Siat7B-related)	*Takifugu rubripes*	n.d.	AJ634461 CAG25682.1		
α-2,6-sialyltransferase ST6GalNAc III (Siat7C) (fragment)	*Takifugu rubripes*	n.d.	AJ634456 CAG25678.1		
α-2,6-sialyltransferase ST6GalNAc IV (siat7D) (fragment)	*Takifugu rubripes*	2.4.99.3	Y17466 CAB44338.1 AJ646869 CAG26698.1	Q9W6U6	
α-2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)	*Takifugu rubripes*	n.d.	AJ646873 CAG26702.1		
α-2,6-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)	*Takifugu rubripes*	n.d.	AJ646880 CAG26709.1		
α-2,8-sialyltransferase ST8Sia I (Siat 8A) (fragment)	*Takifugu rubripes*	n.d.	AJ715534 CAG29373.1		
α-2,8-sialyltransferase ST8Sia II (Siat 8B) (fragment)	*Takifugu rubripes*	n.d.	AJ715538 CAG29377.1		
α-2,8-sialyltransferase ST8Sia III (Siat 8C) (fragment)	*Takifugu rubripes*	n.d.	AJ715541 CAG29380.1		
α-2,8-sialyltransferase ST8Sia IIIr (Siat 8Cr)	*Takifugu rubripes*	n.d.	AJ715542 CAG29381.1		
α-2,8-sialyltransferase ST8Sia V (Siat 8E) (fragment)	*Takifugu rubripes*	n.d.	AJ715547 CAG29386.1		
α-2,8-sialyltransferase ST8Sia VI (Siat 8F) (fragment)	*Takifugu rubripes*	n.d.	AJ715549 CAG29388.1		
α-2,8-sialyltransferase ST8Sia VIr (Siat 8Fr)	*Takifugu rubripes*	n.d.	AJ715550 CAG29389.1		
α-2,3-sialyltransferase (Siat5-r)	*Tetraodon nigroviridis*	n.d.	AJ744806 CAG32842.1		
α-2,3-sialyltransferase ST3Gal I (Siat4)	*Tetraodon nigroviridis*	n.d.	AJ744802 CAG32838.1		
α-2,3-sialyltransferase ST3Gal III (Siat6)	*Tetraodon nigroviridis*	n.d.	AJ626822 CAF25180.1		
α-2,6-sialyltransferase ST6GalNAc II (Siat7B)	*Tetraodon nigroviridis*	n.d.	AJ634462 CAG25683.1		
α-2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)	*Tetraodon nigroviridis*	n.d.	AJ646879 CAG26708.1		
α-2,8-sialyltransferase ST8Sia I (Siat 8A) (fragment)	*Tetraodon nigroviridis*	n.d.	AJ715536 CAG29375.1		
α-2,8-sialyltransferase ST8Sia II (Siat 8B) (fragment)	*Tetraodon nigroviridis*	n.d.	AJ715537 CAG29376.1		
α-2,8-sialyltransferase	*Tetraodon*	n.d.	AJ715539 CAG29378.1		

FIGURE 1K

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
ST8Sia III (Siat 8C) (fragment)	*nigroviridis*				
α-2,8-sialyltransferase ST8Sia IIIr (Siat 8Cr) (fragment)	*Tetraodon nigroviridis*	n.d.	AJ715540 **CAG29379.1**		
α-2,8-sialyltransferase ST8Sia V (Siat 8E) (fragment)	*Tetraodon nigroviridis*	n.d.	AJ715548 **CAG29387.1**		
α-2,3-sialyltransferase (St3Gal-II)	*Xenopus laevis*	n.d.	AJ585762 **CAE51386.1**		
α-2,3-sialyltransferase (St3Gal-VI)	*Xenopus laevis*	n.d.	AJ585766 **CAE51390.1**		
α-2,3-sialyltransferase St3Gal-III (Siat6)	*Xenopus laevis*	n.d.	AJ585764 **CAE51388.1** AJ626823 CAF25181.1		
α-2,8-polysialyltransferase	*Xenopus laevis*	2.4.99.-	AB007468 BAA32617.1	O93234	
α-2,8-sialyltransferase ST8Siα-I (Siat8A;GD3 synthase)	*Xenopus laevis*	n.d.	AY272056 AAQ16162.1 AY272057 AAQ16163.1 AJ704562 **CAG28695.1**		
Unknown (protein for MGC:81265)	*Xenopus laevis*	n.d.	BC068760 **AAH68760.1**		
α-2,3-sialyltransferase (3Gal-VI)	*Xenopus tropicalis*	n.d.	AJ626744 **CAF25054.1**		
α-2,3-sialyltransferase (Siat4c)	*Xenopus tropicalis*	n.d.	AJ622908 **CAF22058.1**		
α-2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)	*Xenopus tropicalis*	n.d.	AJ646878 **CAG26707.1**		
α-2,8-sialyltransferase ST8Sia III (Siat 8C) (fragment)	*Xenopus tropicalis*	n.d.	AJ715544 **CAG29383.1**		
β-galactosamide α-2,6-sialyltransferase II (ST6Gal II)	*Xenopus tropicalis*	n.d.	AJ627628 **CAF29496.1**		
sialytransferase St8Sia I	*Xenopus tropicalis*	n.d.	AY652775 **AAT67042**		
poly-α-2,8-sialosyl sialyltransferase (NeuS)	*Escherichia coli K1*	2.4.-.-	M76370 AAA24213.1 X60598 CAA43053.1	Q57269	
polysialyltransferase	*Escherichia coli K92*	2.4.-.-	M88479 **AAA24215.1**	Q47404	
α-2,8 polysialyltransferase SiaD	*Neisseria meningitidis B1940*	2.4.-.-	M95053 AAA20478.1 X78068 CAA54985.1	Q51281 Q51145	
SynE	*Neisseria meningitidis FAM18*	n.d.	U75650 **AAB53842.1**	O06435	
polysialyltransferase (SiaD)(fragment)	*Neisseria meningitidis M1019*	n.d.	AY234192 **AAO85290.1**		
SiaD (fragment)	*Neisseria meningitidis M209*	n.d.	AY281046 **AAP34769.1**		
SiaD (fragment)	*Neisseria meningitidis M3045*	n.d.	AY281044 **AAP34767.1**		
polysialyltransferase (SiaD)(fragment)	*Neisseria meningitidis M3315*	n.d.	AY234191 **AAO85289.1**		
SiaD (fragment)	*Neisseria meningitidis M3515*	n.d.	AY281047 **AAP34770.1**		
polysialyltransferase (SiaD)(fragment)	*Neisseria meningitidis M4211*	n.d.	AY234190 **AAO85288.1**		
SiaD (fragment)	*Neisseria meningitidis M4642*	n.d.	AY281048 **AAP34771.1**		
polysialyltransferase (SiaD)(fragment)	*Neisseria meningitidis M5177*	n.d.	AY234193 **AAO85291.1**		
SiaD	*Neisseria meningitidis M5178*	n.d.	AY281043 **AAP34766.1**		
SiaD (fragment)	*Neisseria meningitidis M980*	n.d.	AY281045 **AAP34768.1**		
NMB0067	*Neisseria meningitidis MC58*	n.d.	NC_003112 **NP_273131**		

FIGURE 1L

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
Lst	*Aeromonas punctata Sch3*	n.d.	AF126256 **AAS66624.1**		
ORF2	*Haemophilus influenzae A2*	n.d.	M94855 **AAA24979.1**		
HI1699	*Haemophilus influenzae Rd*	n.d.	U32842 **AAC23345.1** NC_000907 **NP_439841.1**	Q48211	
α-2,3-sialyltransferase	*Neisseria gonorrhoeae F62*	2.4.99.4	U60664 **AAC44539.1** AAE67205.1	P72074	
α-2,3-sialyltransferase	*Neisseria meningitidis 126E, NRCC 4010*	2.4.99.4	U60662 **AAC44544.2**		
α-2,3-sialyltransferase	*Neisseria meningitidis 406Y, NRCC 4030*	2.4.99.4	U60661 **AAC44543.1**		
α-2,3-sialyltransferase (NMB0922)	*Neisseria meningitidis MC58*	2.4.99.4	U60660 AAC44541.1 AE002443 AAF41330.1 NC_003112 **NP_273962.1**	P72097	
NMA1118	*Neisseria meningitidis Z2491*	n.d.	AL162755 CAB84380.1 NC_003116 NP_283887.1	**Q9JUV5**	
PM0508	*Pasteurella multocida PM70*	n.d.	AE006086 **AAK02592.1** NC_002663 NP_245445.1	Q9CNC4	
WaaH	*Salmonella enterica SARB25*	n.d.	AF519787 **AAM82550.1**	Q8KS93	
WaaH	*Salmonella enterica SARB3*	n.d.	AF519788 **AAM82551.1**	Q8KS92	
WaaH	*Salmonella enterica SARB39*	n.d.	AF519789 **AAM82552.1**		
WaaH	*Salmonella enterica SARB53*	n.d.	AF519790 **AAM82553.1**		
WaaH	*Salmonella enterica SARB57*	n.d.	AF519791 **AAM82554.1**	Q8KS91	
WaaH	*Salmonella enterica SARB71*	n.d.	AF519793 **AAM82556.1**	Q8KS89	
WaaH	*Salmonella enterica SARB8*	n.d.	AF519792 **AAM82555.1**	Q8KS90	
WaaH	*Salmonella enterica SARC10V*	n.d.	AF519779 **AAM88840.1**	Q8KS99	
WaaH (fragment)	*Salmonella enterica SARC12*	n.d.	AF519781 **AAM88842.1**		
WaaH (fragment)	*Salmonella enterica SARC13I*	n.d.	AF519782 **AAM88843.1**	Q8KS98	
WaaH (fragment)	*Salmonella enterica SARC14I*	n.d.	AF519783 **AAM88844.1**	Q8KS97	
WaaH	*Salmonella enterica SARC15II*	n.d.	AF519784 **AAM88845.1**	Q8KS96	
WaaH	*Salmonella enterica SARC16II*	n.d.	AF519785 **AAM88846.1**	Q8KS95	

FIGURE 1M

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
WaaH (fragment)	*Salmonella enterica SARC3I*	n.d.	AF519772 **AAM88834.1**	Q8KSA4	
WaaH (fragment)	*Salmonella enterica SARC4I*	n.d.	AF519773 **AAM88835.1**	Q8KSA3	
WaaH	*Salmonella enterica SARC5IIa*	n.d.	AF519774 **AAM88836.1**		
WaaH	*Salmonella enterica SARC6IIa*	n.d.	AF519775 **AAM88837.1**	Q8KSA2	
WaaH	*Salmonella enterica SARC8*	n.d.	AF519777 **AAM88838.1**	Q8KSA1	
WaaH	*Salmonella enterica SARC9V*	n.d.	AF519778 **AAM88839.1**	Q8KSA0	
UDP-glucose : α-1,2-glucosyltransferase (WaaH)	*Salmonella enterica subsp. arizonae SARC 5*	2.4.1.-	AF511116 **AAM48166.1**		
bifunctional α-2,3/-2,8-sialyltransferase (Cst-II)	*Campylobacter jejuni ATCC 43449*	n.d.	AF401529 **AAL06004.1**	Q93CZ5	
Cst	*Campylobacter jejuni 81-176*	n.d.	AF305571 **AAL09368.1**		
α-2,3-sialyltransferase (Cst-III)	*Campylobacter jejuni ATCC 43429*	2.4.99.-	AY044156 **AAK73183.1**		
α-2,3-sialyltransferase (Cst-III)	*Campylobacter jejuni ATCC 43430*	2.4.99.-	AF400047 **AAK85419.1**		
α-2,3-sialyltransferase (Cst-II)	*Campylobacter jejuni ATCC 43432*	2.4.99.-	AF215659 **AAG43979.1**	Q9F0M9	
α-2,3/8-sialyltransferase (CstII)	*Campylobacter jejuni ATCC 43438*	n.d.	AF400048 **AAK91725.1**	Q93MQ0	
α-2,3-sialyltransferase cst-II	*Campylobacter jejuni ATCC 43446*	2.4.99.-	AF167344 **AAF34137.1**		
α-2,3-sialyltransferase (Cst-II)	*Campylobacter jejuni ATCC 43456*	2.4.99.-	AF401528 **AAL05990.1**	Q93D05	
α-2,3-/α-2,8-sialyltransferase (CstII)	*Campylobacter jejuni ATCC 43460*	2.4.99.-	AY044868 **AAK96001.1**	Q938X6	
α-2,3/8-sialyltransferase (Cst-II)	*Campylobacter jejuni ATCC 700297*	n.d.	AF216647 **AAL36462.1**		
ORF	*Campylobacter jejuni GB11*	n.d.	AY422197 **AAR82875.1**		
α-2,3-sialyltransferase cstIII	*Campylobacter jejuni MSC57360*	2.4.99.-	AF195055 **AAG29922.1**		
α-2,3-sialyltransferase cstIII Cj1140	*Campylobacter jejuni NCTC 11168*	2.4.99.-	AL139077 **CAB73395.1** NC_002163 **NP_282288.1**	Q9PNF4	
α-2,3/α-2,8-sialyltransferase II (cstII)	*Campylobacter jejuni O:10*	n.d.	-AAO96669.1 AX934427 **CAF04167.1**		
α-2,3/α-2,8-sialyltransferase II (CstII)	*Campylobacter jejuni O:19*	n.d.	AX934431 **CAF04169.1**		
α-2,3/α-2,8-sialyltransferase II (CstII)	*Campylobacter jejuni O:36*	n.d.	AX934436 **CAF04171.1**		
α-2,3/α-2,8-sialyltransferase II (CstII)	*Campylobacter jejuni O:4*	n.d.	AX934434 **CAF04170.1**		
α-2,3/α-2,8-sialyltransferase II (CstII)	*Campylobacter jejuni O:41*	n.d.	-AAO96670.1 -AAT17967.1 AX934429 **CAF04168.1**		
α-2,3-sialyltransferase cst-I	*Campylobacter jejuni OH4384*	2.4.99.-	AF130466 **AAF13495.1** -AAS36261.1	Q9RGF1	
bifunctional α-2,3/-2,8-sialyltransferase (Cst-II)	*Campylobacter jejuni OH4384*	2.4.99.-	AF130984 **AAF31771.1** AX934425 CAF04166.1		1RO7 C 1RO8 A

FIGURE 1N

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
HI0352 (fragment)	*Haemophilus influenzae Rd*	n.d.	U32720 AAC22013.1 X57315 CAA40567.1 NC_000907 NP_438516.1	P24324	
PM1174	*Pasteurella multocida PM70*	n.d.	AE006157 AAK03258.1 NC_002663 NP_246111.1	Q9CLP3	
Sequence 10 from patent US 6503744	*Unknown.*	n.d.	- AAO96672.1		
Sequence 10 from patent US 6699705	*Unknown.*	n.d.	- AAT17969.1		
Sequence 12 from patent US 6699705	*Unknown.*	n.d.	- AAT17970.1		
Sequence 2 from patent US 6709834	*Unknown.*	n.d.	- AAT23232.1		
Sequence 3 from patent US 6503744	*Unknown.*	n.d.	- AAO96668.1		
Sequence 3 from patent US 6699705	*Unknown.*	n.d.	- AAT17965.1		
Sequence 34 from patent US 6503744	*Unknown.*	n.d.	- AAO96684.1		
Sequence 35 from patent US 6503744 (fragment)	*Unknown.*	n.d.	- AAO96685.1 - AAS36262.1		
Sequence 48 from patent US 6699705	*Unknown.*	n.d.	- AAT17988.1		
Sequence 5 from patent US 6699705	*Unknown.*	n.d.	- AAT17966.1		
Sequence 9 from patent US 6503744	*Unknown.*	n.d.	- AAO96671.1		